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The Technique of the Application of Micro-Manometric Methods to Problems of Biology

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I. INTRODUCTION

It was necessary during an investigation of the effect of virus disease on the respiration of potatoes to use micromanometric methods to obtain precise determinations of the rates of respiration of small samples of excised tissue. Several types of equipment are available commercially for this purpose but they are inadequate for the determination of truly micro rates of respiration. The most recent commercially available apparatus possesses ingeniously improved mechanisms for shaking the manometers in the constant temperature water bath but strangely enough the mechanical details affecting the accuracy

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of the experimental results have not been improved over the older types of equipment.

The present report describes certain mechanical features of the manometric equipment and the details of its manipulation which were found necessary in order to obtain accurate and truly micro determinations of the respiration rates of excised plant tissues. Careful attention to certain details of manipulation led to the development of a highly accurate and sensitive procedure which made it possible to measure as little as a few millionths of a gram of oxygen consumed by the experimental tissue with a constant and absolute error of only two millionths of a gram of oxygen. Since the magnitudes of the oxygen consumption determined were of the order of 400 to 600 cubic millimeters, or about 0.57 to 0.85 milligram, the percentage error in the actual determination was very small indeed. As many inquiries have been received concerning the technical aspects of the procedure, it appeared advisable to describe the apparatus and its manipulation in considerable detail for the convenience of other workers.

For many years various types of micro-manometric methods have been used by animal physiologists and biochemists, and these methods have become routine procedures in many branches of their studies. Plant physiologists, however, have been slow to profit by the experience of workers in these related fields. This is probably due to fear of the unknown. Since their work largely concerns the activities of entire plants, many plant physiologists are of the opinion that nothing can be learned by studying excised tissue. The criticism that excised tissue is abnormal can be refuted at least partially, on the strength of the fundamental work accomplished by animal physiologists on such tissue. If the science of animal physiology had depended solely on entire organisms observed under normal conditions, it would not be in its present high stage of development. As plants do not depend on such intricate organizations of tissue as do animals, it is logical to assume that excision of plant tissue will induce even less abnormal conditions than excision of animal tissue.

Strictly speaking, any application of physico-chemical methods to biology is made at the expense of a completely normal environment. Hence the application of such methods must always be made with care, and the results intelligently analyzed. But rejection of such methods on this account betrays limited knowledge of the fundamental contributions made with them in fields closely related to plant physiology.

The inherent accuracy of micro-manometric technique is very great. Under ideal conditions, an experienced operator can detect changes in volume corresponding to only 0.000,002 gram of oxygen with an accuracy of 90 per cent. Larger amounts can be detected with greater accuracy, since the error tends to be absolute in magnitude rather than a percentage of the total. Routine procedure under a variety of conditions, which includes simplified calibration factors and a permissible variability in manipulation, is predictably accurate to 98 per cent of the measured changes in gas volumes. It is difficult to draw graphs of families of numerical quantities which differ by less than two percent, hence any difference that is apparent with ordinary graphing procedures becomes a "significant" difference. This "significance" means only that such observed differences are real and not errors of the apparatus. The

"biological significance," of course, is another matter and is dependent on the nature of the research, and on the accuracy of the sampling technique.

The significance of differences raises a perplexing biological problem. It is assumed, casually and without contemplation, that a significant difference is something of biological importance, but a survey of the experimental results in any field of science shows that almost invariably the limits of a significant difference are not biological in nature, but are merely the smallest quantities that can be measured with certainty. For example, a significant difference in respiratory rates determined by acid-alkali titration in the air must certainly be very much larger than when determined by the finest of micro-manometric techniques. Our knowledge of plant physiology is so meager that we cannot define what is a physiologically significant difference in respiration rates, and it is therefore necessary to place it arbitrarily at two percent since this is the smallest difference that can be determined with certainty in routine procedures which also involve errors of sampling.

II. Apparatus

A. Water bath

The accurate control of the temperature of the water bath demands that the bath contain at least two cubic feet of water. It is difficult to maintain the temperature of a smaller volume with adequate accuracy, while larger volumes are difficult to stir with sufficient rapidity and evenness.

The tank need not be insulated unless it is to be used for very high temperatures, because the accuracy of the temperature control described below depends on rapid equilibrium between efficient heating and efficient cooling. At temperatures of 37 degrees Centigrade or lower, insulation is neither necessary nor desirable.

Most of the commercially available stirring devices are inadequate to maintain a proper agitation of the water. It was found necessary to mount two stirring fans on each of two shafts. The fans were made of 16-gage brass, and were 5.5 inches in diameter. The tilt of the blades was adjusted experimentally until the maximum violence of stirring was obtained which would not throw the water out of the tank. The rate of stirring can be much greater without splashing if the water is forced downward.

B. Oscillator

If the gas reactions to be observed take place in liquids, it is necessary that the manometers be shaken. It has been shown by experiment that gases do not maintain a constant equilibrium with liquids unless the vessels are oscillated at a rate of at least 120 excursions per minute. It is absolutely necessary that all joints of the shaking device be so accurately made that there is no jar, or click, as the manometers reverse direction. If any jerk occurs, the menisci are very hard to read while in motion, and the liquids will tend to creep out of their respective compartments in the reaction vessels. *This is one of the most critical points in the construction of the apparatus.* If the shaker is not perfectly constructed, many experiments will be ruined. The smoothness of its operation is greatly enhanced if the moving parts of the shaker be kept liberally oiled with a heavy machine oil.

The modern type of oscillator which rotates the manometers through a small arc rather than sways them through a vertical angle, is very satisfactory, since the danger of splashing of the liquids in the experimental vessels is lessened, and the menisci may be read more easily while the manometers are in motion.

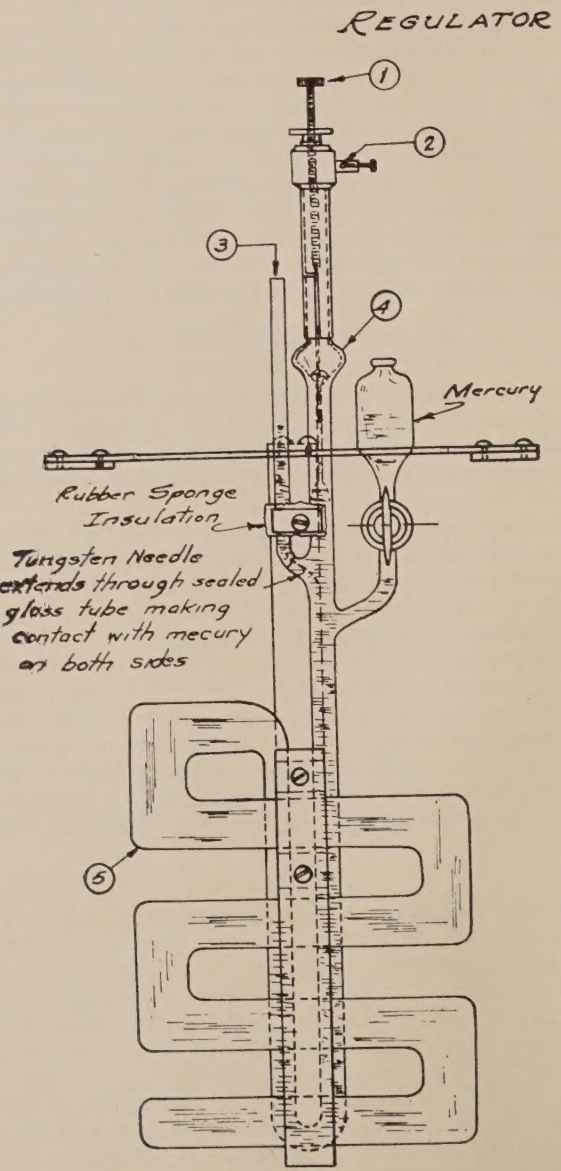

FIGURE 1. The thermoregulator. The tungsten needle is adjusted at 1 to some level in the capillary below 4. The electrical contacts are at 2 and 3. The swelling at 4 gives a firm rest for the regulator cap.

C. THERMOREGULATOR

Bimetal thermoregulators are not satisfactory. Their response to temperature change is too slow, and their electrical contacts are difficult to maintain in perfect condition.

A mercury thermoregulator of the type illustrated in Figure 1 is especially satisfactory. The large, bent tube is made of glass and is about 0.75 inch in diameter. It is filled with toluene or carbon tetrachloride. The smaller vertical tube is filled with clean mercury from the tip of the regulator needle to well beyond the lower bend. The reservoir on the side, and the stopcock below it, allow changes to be made conveniently for the establishment of various temperatures.

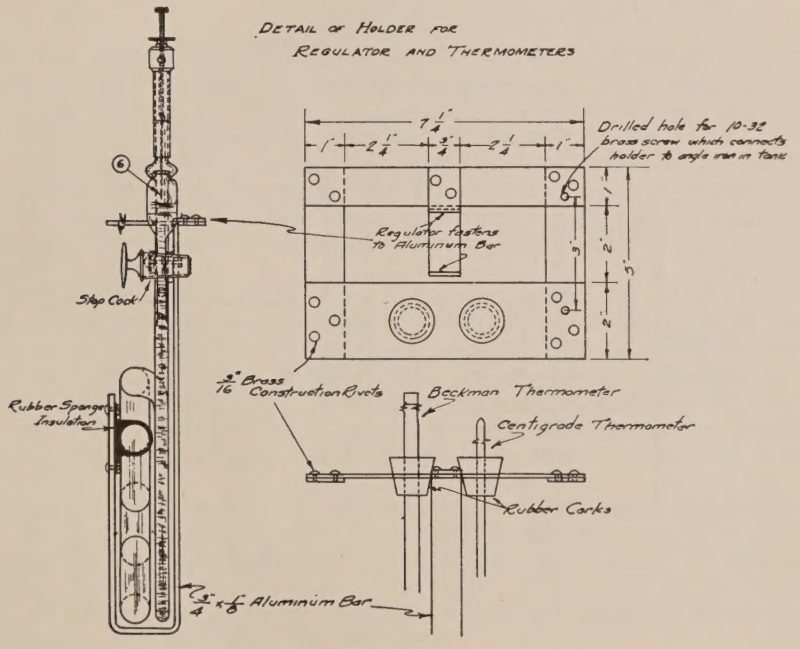

FIGURE 2. The holder for the thermoregulator.

The regulator cap may be purchased from the Pantechnical Mfg. Co., 3063 Adeline St., Berkeley, California. The glass parts of the apparatus can be made by any competent glass blower. The tip of the regulator needle is tungsten and it fits into the capillary below the point marked 4 in Figure 1. The level of the mercury should be kept within this capillary. It is necessary that the tip of the needle be very clean. It can best be cleaned, and at the same time sharpened, by heating it red hot and then drawing it rapidly against a stick of sodium nitrite. A pipe-stem cleaner may be used to remove the toluene or carbon tetrachloride that may collect above the mercury meniscus after the regulator has been filled. Once this surface liquid has been removed, no further trouble from this source is encountered.

The mounting for the thermoregulator supports a Centigrade and also a Beckmann thermometer. The mounting is attached to an

angle-iron attached to one side of the water bath, or suspended from above. The details of the mounting are shown in Figures 1 and 2. Wires connect 2 and 3 in Figure 1 to the proper terminals of the vacuum-tube relay described below. If an electrical shock is obtained when the regulator cap is touched, the terminals of the power input to the relay should be reversed. To avoid an electrical shock, the polarity of the regulator cap must be that of the grounded terminal of the generator which supplies the current. However, the electrical shock cannot be received unless the operator is himself grounded by standing on a cement floor or by contact with a water or gas main.

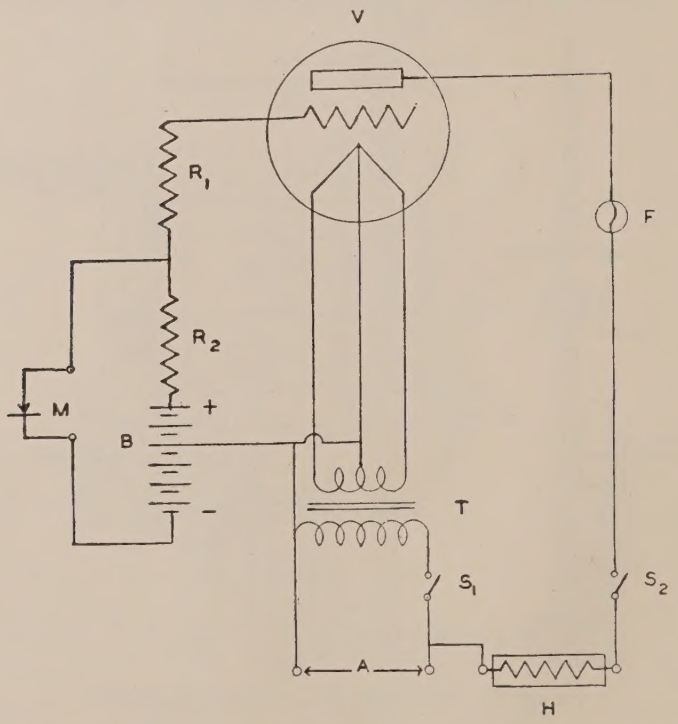

FIGURE 3. A, 110-volt alternating current; B, two standard size 4½ volt '.C–' batteries; M, contacts of toluol-mercury thermoregulator; F, 5-ampere auto type fuse; H, submerged heater unit; R^1, one megohm; R_2, 100,000 ohms; S_1 and S_2, switches; T, five-volt 25-watt center tapped transformer; V, Thyratron tube Fg-27 or Fg-57.

D. ELECTRIC RELAY

An especially critical part of the apparatus is the electric relay controlling the temperature of the water bath. Many types of commercially available relays were investigated and, in general, were found to be unsatisfactory. These relays have too great a time lag in their response because of the mechanical construction of their moving parts and because they require too heavy a current for their activation. The most satisfactory type of relay is that designed by Schmitt and

Schmitt (1931) using the General Electric Tyratron tube FP57. The details of the construction are shown in Figure 3. A number of these relays have been used in the present study and they always functioned perfectly over long periods of time. The tube should be heated 15 minutes before its output is turned on in order to avoid an initial overload on the tube. If the voltage heating its filament drops more than 10 percent, the durability of the tube is impaired or the tube may be completely ruined. It is advisable to connect an automatic cut-out in the input circuit which will shut off the current if the voltage drops. If the voltage on the input circuit should be cut off, the tube would be ruined if it is allowed to carry a load in its output circuit until it is again thoroughly heated.

E. HEATER

The heater in the tank should be the 400-watt Lo-lag type manufactured by the American Instrument Company. It is essential for accurate temperature control that the heater be of low wattage and that it possesses a comparatively large surface exposed to the water. The method of temperature control described above will consistently control the temperature of the water bath so accurately that no movement of the mercury column in a Beckmann thermometer can be detected. Under optimum conditions the temperature should not vary more than 0.005° C.

Most experiments with plant tissue are carried out at 25° C. If the room temperature approaches this temperature within three or four degrees it is necessary to cool the bath by circulating trap water through copper coils. The flow of water must be controlled accurately by a good faucet or pinch cock. Usually a very small flow will be sufficient. During very warm weather it may be necessary to circulate the cooling stream through a copper coil immersed in ice water or enclosed in an ice chest.

On the other hand, if the operator wishes to maintain a temperature of 37° C. during cold weather, it may be necessary to add a constant supply of heat to the system. This can best be done by a 600-watt heater controlled by a rheostat. The output of this heater is controlled to a constant value by the rheostat so that the regulator heater can maintain the temperature desired.

F. EFFECT OF TEMPERATURE VARIATIONS

Since the volume of a gas is proportional to its absolute temperature, it follows that significant changes in the temperature of the water bath must be avoided. This is particularly important if manometers having one end open to the air are used. The error, in percentage of the total gas present, depends on the magnitude of the temperature change, and the absolute magnitude of the error in terms of actual gas volume depends on the volume of the vessel. Hence a change of 0.1° C. from an Absolute Temperature of 300° introduces an error of 0.03 percent. The absolute magnitude of the error for vessels of 10 milliliters capacity would be three cubic millimeters and for a vessel of 30 milliliters capacity the error would be nine cubic millimeters. Since routine micro-manometric technique records gas changes of the order of 0.1 cubic

millimeter, it is seen that the temperature control must be accurate indeed.

Temperature variations are less important if differential manometers are used, and this is one of the arguments in favor of their use.

TABLE 1. *Effect of temperature variations on the magnitude of the calibration factor of the differential manometer with vessels of a volume of about 5 milliliters.*

Volume in Cubic Millimeters of Compensating Vessel	Volume in Cubic Millimeters of Experimental Vessel	Absolute Temperature	Calibration Factor K
5000	5000	298.0	0.8116
5000	5000	298.1	0.8114
5000	5000	298.3	0.8108
5000	5050	298.0	0.8170
5000	5050	298.0	0.8167
5000	5050	298.3	0.8161
5000	5100	298.0	0.8223
5000	5100	298.1	0.8220
5000	5100	298.3	0.8215
5000	5150	298.0	0.8276
5000	5150	298.1	0.8273
5000	5150	298.3	0.8269

TABLE 2. *Effect of temperature variations on the magnitude of the calibration factor of the differential manometer with vessels of a volume of about 10 milliliters.*

Volume in Cubic Millimeters of Compensating Vessel	Volume in Cubic Millimeters of Experimental Vessel	Absolute Temperature	Calibration Factor K
10,000	10,000	298.0	1.1126
10,000	10,000	298.1	1.1122
10,000	10,000	298.3	1.1111
10,000	10,100	298.0	1.1214
10,000	10,100	298.1	1.1210
10,000	10,100	298.3	1.1202
10,000	10,200	298.0	1.1302
10,000	10,200	298.1	1.1298
10,000	10,200	298.3	1.1291
10,000	10,300	298.0	1.1390
10,000	10,300	298.1	1.1386
10,000	10,300	298.3	1.1379

Strict physical theory eliminates the temperature effect entirely, but in actual practice the two components of a differential system are never perfectly symmetrical in their behavior, and therefore even with differential manometers, temperature variations are not without some

effect. The data in Tables 1, 2, and 3 indicate the effects of small changes of temperature on the calibration factor of a differential manometer with a capillary of 0.400 square millimeters in area. Since the calculations are based on vessels containing no liquids, the factor K has no reference to a particular gas. It is seen that variations of 0.3° C. cause calibration errors to appear in the third decimal place.

TABLE 3. *Effect of temperature variation on the magnitude of the calibration factor of the differential manometer with vessels of a volume of about 20 milliliters.*

Volume in Cubic Millimeters of Compensating Vessel	Volume in Cubic Millimeters of Experimental Vessel	Absolute Temperature	Calibration Factor K
20,000	20,000	298.0	1.7866
20,000	20,000	298.1	1.7860
20,000	20,000	298.3	1.7845
20,000	20,200	298.0	1.8024
20,000	20,200	298.1	1.8018
20,000	20,000	298.3	1.8005
20,000	20,400	298.0	1.8181
20,000	20,400	298.1	1.8175
20,000	20,400	298.3	1.8163
20,000	20,600	298.0	1.8334
20,000	20,600	298.1	1.8333
20,000	20,600	298.3	1.8321

G. MANOMETERS

1. *Types*

Single manometers. Single manometers of the type shown in Figure 4 were developed by Barcroft (1902) for the determination of gas exchanges of small quantities of blood. This form of manometer, perfected by Warburg (1930), is now in general use in the form pictured in Figure 5. The advantages of the single manometer are that it is relatively inexpensive, requires but a single reaction vessel, has a simpler and more accurate calibration equation, and its narrowness permits a larger number to be in operation at one time in the bath. The disadvantages are that the constant volume type cannot be read while in motion except in especially constructed equipment and it is affected by changes in barometric pressure. The inconvenience incurred by changes in atmospheric pressure is very great, especially if the experiment continues over a considerable period of time. Further, the barometric pressure corrections cannot depend on values obtained from a column of mercury since its specific gravity is about 13 times that of the liquid in the capillary of the manometer. This means that the manometer is about 13 times as sensitive to atmospheric pressure changes as is mercury which makes it impossible to read a barometer accurately enough to make satisfactory corrections. In practice, it is necessary to use a blank manometer as a barometer in order that

the changes in atmospheric pressure can be observed with sufficient accuracy. Single manometers may not be used for the determination of volumetric changes with the highest accuracy.

Differential manometers. The advantage of the differential manometer is that it is a closed system and therefore independent of changes

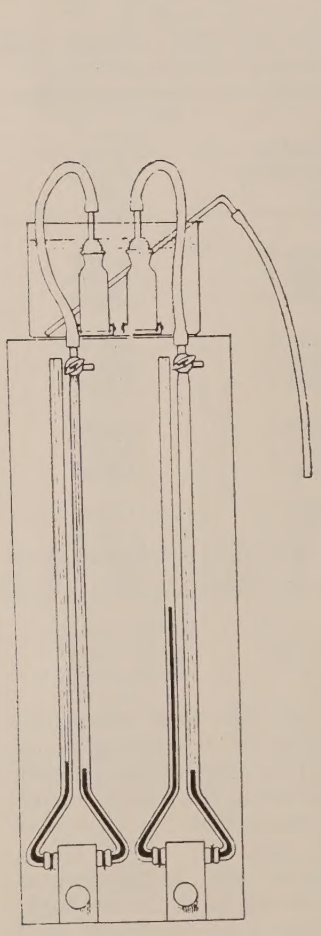

Fig. 4

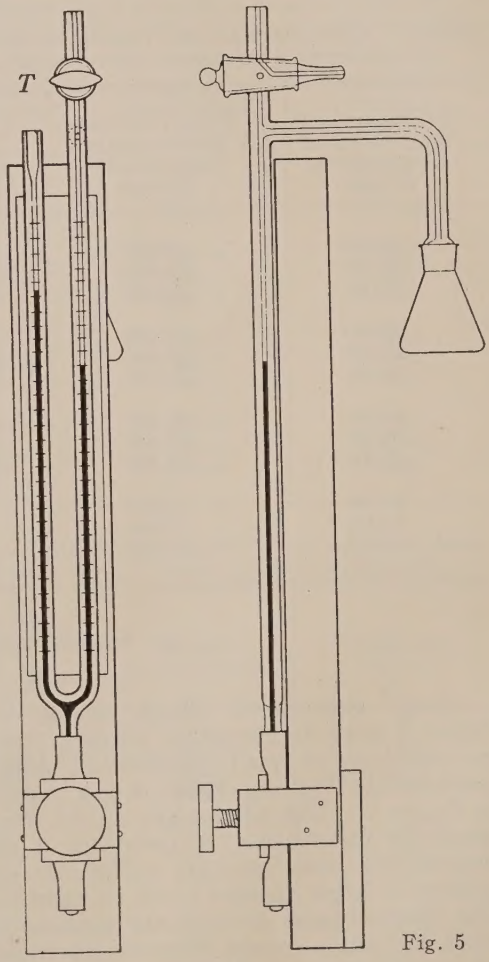

Fig. 5

FIGURE 4. An old type of crude, constant volume single manometer described by Barcroft (1902).

FIGURE 5. The commercially available modern type of constant volume single manometer. The figure is from Dixon (1934).

in atmospheric pressure. The disadvantages are its complicated calibration equation, its expensive construction, the necessity for two reaction vessels, and the fact that its width limits the number that can be operated simultaneously in the water bath. A further difficulty

is that no satisfactory type is available commercially. Figure 6 illustrates all necessary details which will enable any competent glass blower to undertake their construction. This type of manometer is necessary for observations of the highest accuracy.

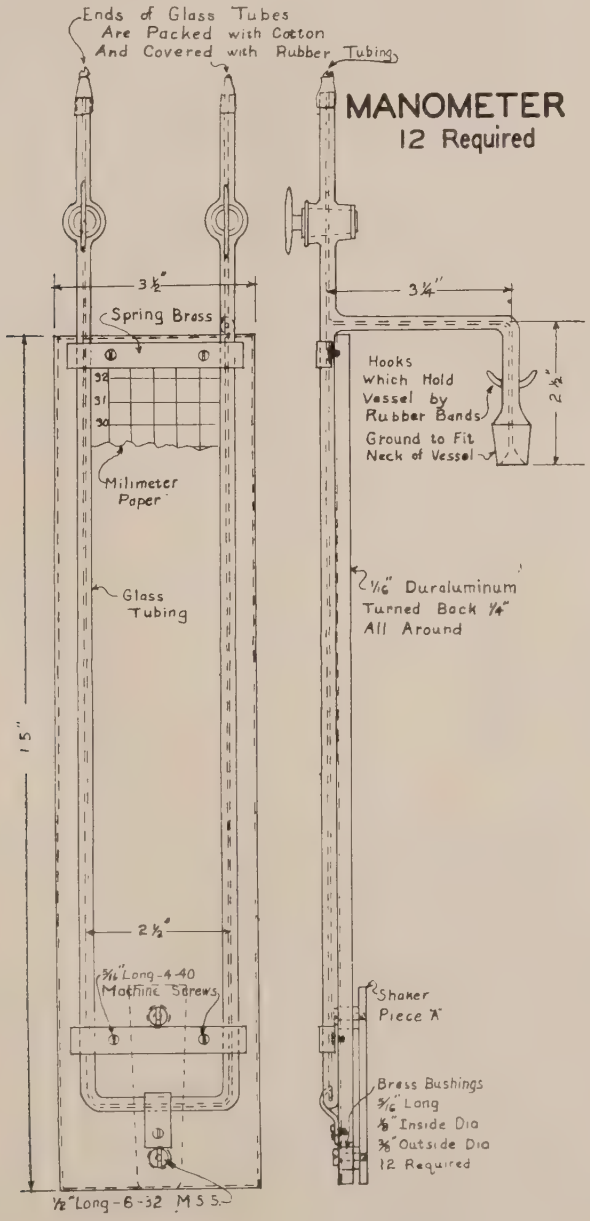

FIGURE 6. A modern type of differential manometer.

2. *Capillaries*

Satisfactory manometers of either the single or differential type demands that the capillaries used in their construction be of small and uniform bore. It is necessary to examine large quantities of tubing in order to find acceptable capillaries. The area of the cross section of the capillary should be of the order of 0.3 to 0.4 square millimeter. *In no case may it exceed 0.5 or significant errors will be introduced into the simplified calibration formulae presented below.* The area of the cross section of the capillary may be found by drawing a length of mercury into the capillary, measuring its length in millimeters and then weighing the mercury on a chemical balance. From the specific gravity of mercury, the length of the column, and its weight, its volume can be calculated, and the area of the capillary is easily obtained.

The evenness of the diameter of the bore is observed by moving a column of mercury of about 100 millimeters in length along the tube

TABLE 4. *The effect of the area of the cross section of the capillary on the magnitude of the calibration factor.*

Volume of Each Vessel in Cubic Millimeters	Area of Capillary in Square Millimeters	Calibration Factor K
5,000	0.200	0.5563
5,000	0.400	0.8114
5,000	0.600	1.1151
10,000	0.200	0.8933
10,000	0.400	1.1126
10,000	0.600	1.3560
20,000	0.200	1.5853
20,000	0.400	1.7866
20,000	0.600	1.9984

and measuring the changes its length undergoes. The successive measurements should not differ by more than 2 percent. The selected tubing should be carefully cleaned before it is given to the glass blower in order to avoid roughness developing on the inner surface when heated. The cleaning is best done by drawing hot nitric acid through the tube, then distilled water, and finally hot filtered air.

The size of the capillary has a great influence on the magnitude of the calibration factor. Table 4 presents data which show that the area must be determined to ± 0.01 square millimeter in order to permit the calculation of a calibration factor of sufficient accuracy.

3. *Manometer Liquids*

The liquid most commonly used in the manometer is Brodie's solution which is made up by adding 23 grams of sodium chloride, 5 grams of sodium choleate and a few drops of thymol to 500 milliliters of distilled water. The specific gravity of this solution is such that a

column of about 10,000 millimeters equals a pressure of one atmosphere. This value greatly simplifies the calculation of the calibration factor. The use of this liquid has the disadvantage of fouling the capillary which makes it necessary to clean the manometer frequently. Caproic acid also is used occasionally. It has a specific gravity which makes a column of 11,160 millimeters equal to one atmosphere. The author prefers to use triple-distilled kerosene, stained with Sudan III. The specific gravity of each sample must be determined by weighing 10 milliliters in a pycnometer. Usually, one atmosphere equals a column of about 13,000 millimeters. Kerosene may not be used in constant volume manometers, since it dissolves the rubber of the reservoir.

4. Manometer Scale

Most commercially available manometers have a millimeter scale engraved on the tubing. This hinders accurate reading of the meniscus. The menisci can be read more accurately if the capillaries are smooth and millimeter graph paper is mounted behind the tubes.

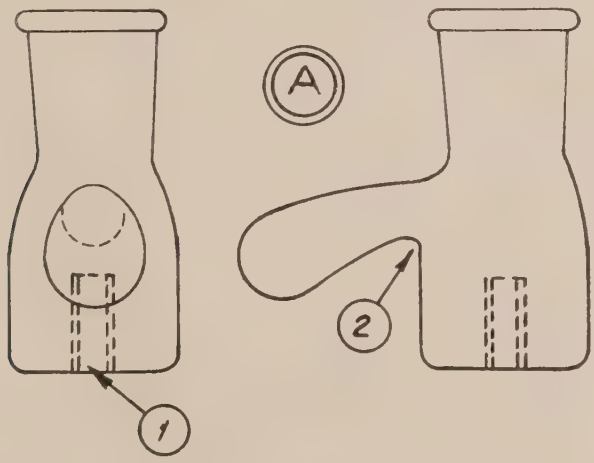

FIGURE 7. Natural size of highly sensitive vessels for liquid reactions.

H. VESSELS

1. Construction

The connecting joints of the manometers are standard taper 14/20 with a two-millimeter capillary. The stopcocks also have a bore of 2 millimeters.

Numerous types of vessels have been described in the literature. Many of these are available in various sizes in order that the sensitivity of the system can be adjusted to the rate of the reaction to be studied. For many types of observations, it will be found necessary to design vessels especially adapted to the nature of the experiment. The types most frequently used are shown in Figures 7 to 11, inclusive. If the reaction takes place in a liquid, and if a high degree of sensitivity is desired, types A, B, or C, shown in Figures 7, 8, and 9 are used. Type

Fig. 8 Fig. 9

FIGURE 8. Natural size of highly sensitive vessels for dry or liquid samples.
FIGURE 9. Natural size of reaction vessels of the highest sensitivity.

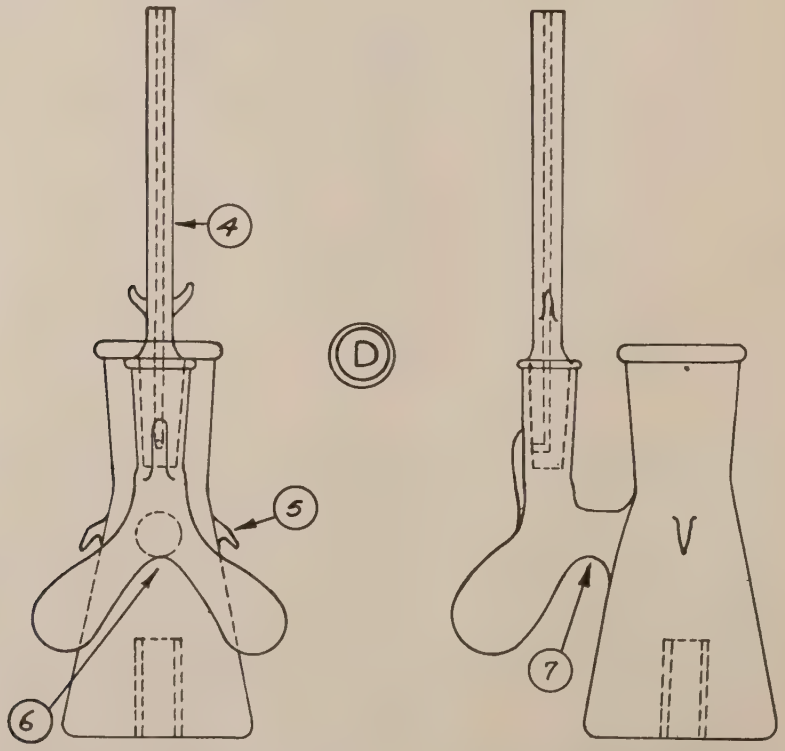

FIGURE 10. Natural size of vessels of moderate sensitivity. The angle at 7 must be such that liquids can be tipped from the side arm into the reaction chamber. 4 is a vent, permitting aeration of the vessel. Rubber bands hold the vessel firmly to the manometer by attaching to 5.

A has an inset containing a few drops of alkali to absorb carbon dioxide. The side arm permits an experimental liquid to be tipped into the reaction vessel at any time. It is essential that the angle of the side arm at 2 be such that the liquid in the side arm can be easily tipped out. In type B, the alkali is put in the side arm. In this instance, the angle indicated at 3 is less than that at 2 for vessels of the A type. Type B

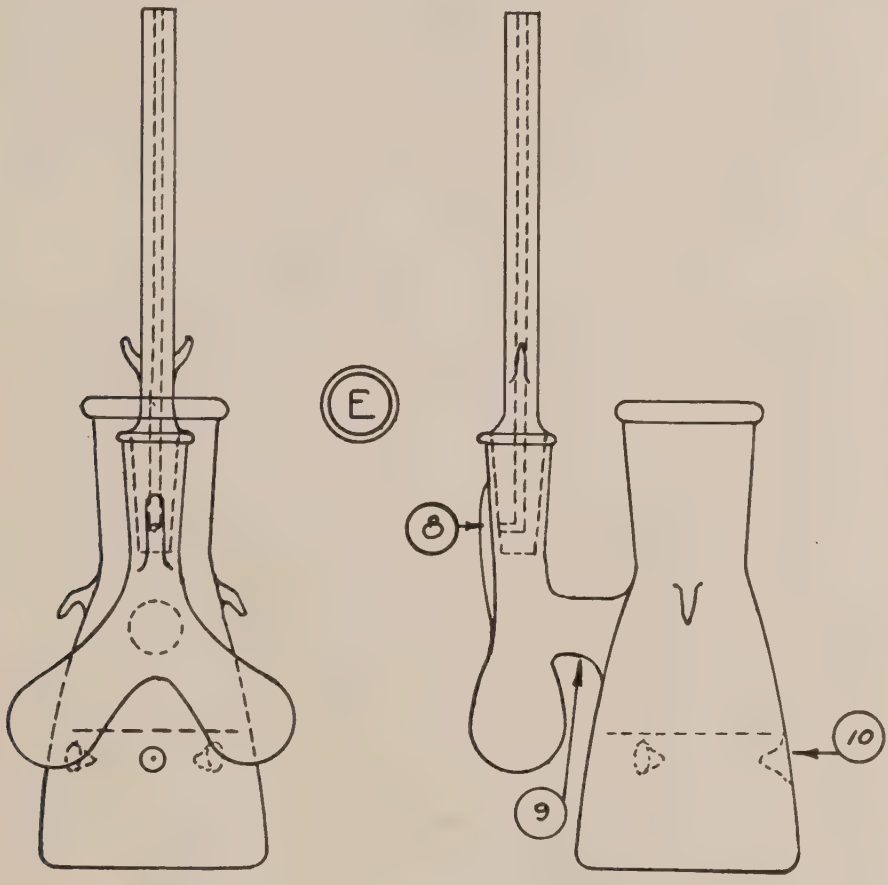

FIGURE 11. The identations at 10 support a wire screen platform.

can be used conveniently for portions of leaves, flower buds and simple types of samples. Type C, illustrated in figure 9, is designed for maximum sensitivity. To reduce its volume, no provision is made for an alkali chamber. A small piece of filter paper is moistened with alkali, and is then held up in the mouth of the manometer joint by a short piece of bent wire thrust upwards into the capillary.

Type D, shown in Figure 10, also is used for observation on liquid samples. The outlet, 4, permits the chamber to be swept out and filled with an atmosphere of any desired composition, and by a half turn the

vessel is sealed. The double side arm permits an acid and alkali to be present for the determination of the respiratory quotient as described below. In the event that an experimental liquid is to be tipped into the sample, the alkali is placed in the inset and the liquid in one of the side arms. If used in this manner the angle at 7 must be such that the liquid can be tipped into the larger compartment.

Type E, in Figure 11, is designed especially for use with leaves, small fruits, and similar samples. Three indentations are made, as shown at 10, which support a wire screen platform. The tissue rests on this platform, and a little water is placed in the space beneath in order that the atmosphere within the vessel be quickly and continuously saturated with water vapor. The alkali is put in one of the side arms, and if the respiratory quotient is desired acid is put in the other. Since these liquids are not to be tipped into the reaction compartment, the angle at 9 may be made very small.

2. Types of Observations

If the oxygen uptake is to be determined, it is necessary that alkali be present in some compartment of the vessel to absorb carbon dioxide as it is produced. Carbon dioxide may be determined by observing pressure changes produced by a duplicate sample in the absence of alkali, and the proper calculation then made by a comparison of the two rates. This method of determining carbon dioxide and resulting respiration quotients is to be preferred in all experiments which permit an accurate sampling technique. If this is not possible, then the carbon dioxide must be determined on the same sample used to determine the oxygen absorption. This is done by first obtaining the rate of oxygen consumption, and then releasing the carbon dioxide that has been produced during the same period by tipping phosphoric acid into the alkali and noting the pressure change. While this procedure sounds simple enough, it is difficult and tedious to carry out in the laboratory. The amount of carbonate in the alkali at the beginning of the experiment must be determined by another manometer which has been handled in exactly the same manner. In liquid samples, the carbon dioxide held in the liquid also must be determined at the beginning and at the end of the experiment. Accurate timing of these observations is necessary, especially if dry samples are used, in order to permit the proper calculations. This procedure for determining carbon dioxide and respiratory quotient should never be applied if the method using duplicate samples can be used.

Many different types of physiological reactions can be followed by manometric technique. For details, the worker should consult the literature of the specific problem to be investigated.

3. Relation of Volume to Calibration Factor

The size of the gas space in the reaction chamber affects directly the sensitivity of the system and this sensitivity is reflected by the magnitude of the calibration factor. For single manometers, the smaller the vessel the more sensitive is the system, and the smaller is the calibration factor. This is true for the constant volume type of single manometer as well as for the differential type when the com-

pensating vessel is open to the air. The mathematical calculations are described below.

Reference to Table 5 and Figure 12 shows that differential manometers are limited in the degree of sensitivity that can be obtained by

TABLE 5. *The effect of the gas space volume of the vessels in the sensitivity of a differential manometer with the area of the capillary of 0.40 square millimeter.*

Volume of Each Vessel in Cubic Millimeters	Calibration Factor K
40,000	3.171
20,000	1.786
10,000	1.112
6,000	0.865
5,000	0.811
3,500	0.747
3,000	0.735
2,000	0.745
1,500	0.790
1,000	0.916
400	1.590

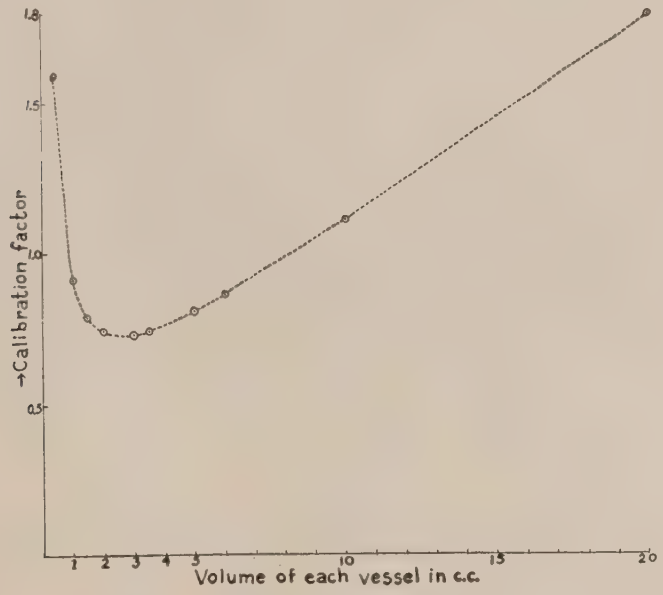

FIGURE 12. The relation of the volume of the vessels to the sensitivity of the differential manometer.

diminishing the size of the vessels. It is impractical to diminish the size of the vessels to less than 5 milliliters. As the size of the vessels increases above 5 milliliters, there is an almost linear relationship between the size of the vessel and the sensitivity of the system.

III. CALIBRATION

A. MEASUREMENTS

1. Capillaries

The volume of a vessel includes the gas space in the arm of the manometer to which it is attached and that of the capillary down to the level of the meniscus. Since calibration factors must be calculated for various vessels and for vessels containing various volumes of samples and liquids, it is convenient to record permanently the manometric component of the total volume, so that new calculations can be made at any time. The volume of the arm and capillary is obtained by filling the space with mercury and then weighing the amount used. By carefully manipulating the manometer by tipping it in the hand before

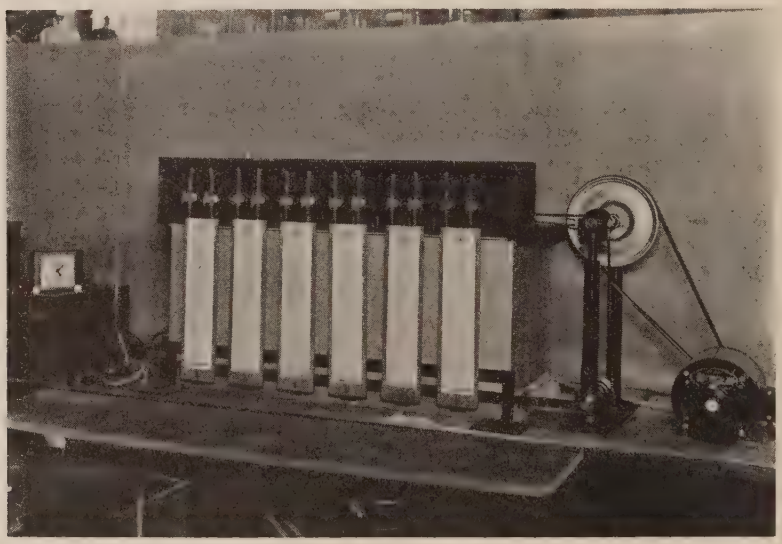

FIGURE 13. Assembled apparatus for micro-manometric determinations. A removable, black painted cover has been placed over the water bath to exclude light from the reaction vessels. The black box at the left is the thyratron temperature control. The flickering of the pilot light mounted in its face indicates the constancy of the temperature control.

the liquid has been put in the capillary, the proper amount of mercury can be added. The volume determined must be that extending to a permanently indicated point on the arm just above its connecting ground joint.

2. Vessels

The volume of the vessel is obtained by filling it with mercury until the level of the mercury just reaches the point used to determine the volume of the capillary when the vessel is firmly in place on the manometer. Table 6 presents data showing the degree of accuracy necessary in the determination of the gas-space volume. It is seen that errors

of 1 percent of the total volume are permissible. This degree of accuracy is easily obtainable for the larger vessels, but careful technique is required when calibrating the smaller types.

TABLE 6. *The effect of variations of the gas space in the vessels on the accuracy of the calibration factor of a differential manometer with a capillary of 0.40 square millimeter.*

Volume in Cubic Millimeters of Compensating Vessel	Volume in Cubic Millimeters of Experimental Vessel	Calibration Factor K	Percent Error in K
5,000	5,000	0.811	
5,000	5,050	0.815	0.62
5,000	5,100	0.821	1.24
5,000	5,150	0.826	1.85
10,000	10,000	1.112	
10,000	10,100	1.121	0.81
10,000	10,200	1.129	1.53
10,000	10,300	1.138	2.34
20,000	20,000	1.786	
20,000	20,200	1.801	0.86
20,000	20,400	1.818	1.75
20,000	20,600	1.832	2.58

3. *Manometer Liquid*

The specific gravity of the liquid used in the manometer must also be known. Accurate values may be obtained by weighing 10 milliliters of the liquid in a pycnometer. From this weight, the column in millimeters equalling a pressure of one atmosphere is calculated. The data in Table 7 show that errors of 1 percent are permissible.

TABLE 7. *The effect of variations in the specific gravity of the liquid in the capillary on the accuracy of the calibration factor of a differential manometer with a capillary with an area =0.40 square millimeter.*

Size of Each Vessel in Cubic Millimeters	Column of the Liquid in mm. to Equal 1 Atmosphere	Calibration Factor K
5,000	10,000	0.898
5,000	10,100	0.894
5,000	10,200	0.890
10,000	10,000	1.320
10,000	10,100	1.310
10,000	10,200	1.302
20,000	10,000	2.217
20,000	10,100	2.199
20,000	10,200	2.181

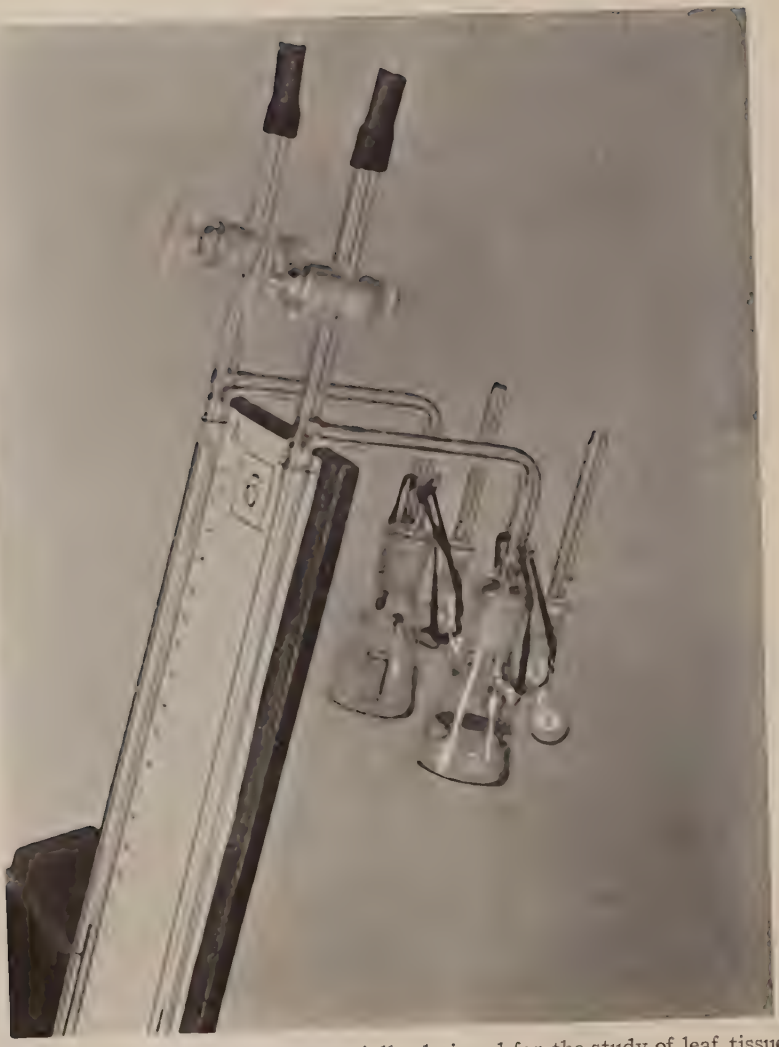

FIGURE 14. Reaction vessels especially designed for the study of leaf tissue. A wire screen supports the sample over a pool of water.

B. CALCULATIONS

1. Single Manometer

The single manometer of the constant volume type has a comparatively simple and easily derived calibration factor. By multiplying the observed difference in height of the menisci of the two limbs of the manometer by this factor, the change in volume of the gas under observation is obtained. It will be seen from the manner by which this factor was derived by Warburg (1930), that the values obtained are corrected for standard pressure and temperature. The following numerical quantities enter into the calculation:

x = cubic millimeters of gas evolved during the experiment (x has a negative sign if gas is absorbed).
h = difference in millimeters in the heights of the menisci in the limbs of the manometer.
V_g = volume of gas in cubic millimeters in the experimental vessel at the beginning of the experiment.
V_f = Volume of liquid in cubic millimeters in the experimental vessel.
T = Absolute temperature.
P = initial atmospheric pressure expressed in millimeters of the liquid in the capillary of the manometer.
P_o = millimeters of the liquid in the capillary equaling a pressure of one atmosphere.
$_p$ = vapor pressure of water at the temperature T.
α = Bunsen solubility coefficient of the gas which is changing in volume.

Since P_{-p} is the pressure within the vessel which is exerted on the non-aqueous gas at the beginning of the experiment, and since the temperature is T, the initial volume of gas under standard conditions is

$$V_g \frac{273}{T} \frac{P-p}{P_o} \tag{1}$$

A part of the gas is dissolved in the liquids present in the vessel. The volume of dissolved gas is

$$V_f \alpha \frac{P-p}{P_o} \tag{2}$$

The total amount of gas initially present is the sum of (1) and (2).

$$\left(V_g \frac{273}{T} \frac{P-p}{P_o} \right) + \left(V_f \alpha \frac{P-p}{P_o} \right) \tag{3}$$

After a reaction has changed the gas pressure within the vessel, the new volume of gas in the space is equal to that originally present corrected by the new pressure, hence (1) becomes

$$V_g \frac{273}{T} \frac{P-p+h}{P_o} \tag{4}$$

The amount of gas dissolved in the liquid must also be corrected for the new pressure, hence (2) becomes

$$V_f\alpha \frac{P-p+h}{P_o}$$

The total volume of gas finally present is equal to that originally present plus x and therefore

$$\left(V_g \frac{273}{T} + V_f\alpha\right)\left(\frac{P-p}{P_o}\right) + x = \left(V_g \frac{273}{T} + V_f\alpha \frac{P-p+h}{P_o}\right) \quad (5)$$

Solving (5) for x in terms and factoring for h, we obtain

$$x = h\left(\frac{V_g \frac{273}{T} + V_f\alpha}{P_o}\right) \quad (6)$$

The term within the brackets becomes the calibration constant for the conditions imposed by the environment.

2. *Differential Manometer*

When used differentially. The differential manometer can be used conveniently to determine the volumetric difference between two rates of gas change. Barcroft (1908) adapted this procedure for the comparison of the gas exchange of arterial and venous blood. The derivation of the calibration formula for this type of observation is due to Sir Robert S. Ball and is included in the paper of Barcroft. The following numerical quantities are involved in its derivation:

V_g = original volume of gas in cubic millimeters in each vessel (it is assumed that V_g is the same in both of the vessels).
P = millimeters of the liquid in the capillary equalling one atmosphere.
xV_g = gas absorbed in vessel A.
yV_g = gas absorbed in vessel B.
p = millimeters of movement in one limb of the manometer.
d = diameter of the capillary.
S = area of the cross section of the capillary in square millimeters.
h = difference in the height of the menisci in the limbs of the manometer.

The volume of gas in vessel A would be V_g (1–x) if it were at the original pressure. But the actual pressure is P_{-p}, therefore the volume of gas in A actually is

$$\frac{V_g\ (1-x)\ P}{P-p} \quad (7)$$

Similarly, the actual volume of gas in vessel B is

$$\frac{V_g\ (1-y)\ P}{P+p} \quad (8)$$

The difference between (8) and (7) must be the volume of the capillary between the heights of the menisci in the two limbs of the manometer, therefore

$$\frac{V_g\ (1-y)\ P}{P+p} - \frac{V_g\ (1-x)\ P}{P-p} = 2\ Sp \tag{9}$$

The desired quantity is the difference of the final volumes of A and B at the original pressure. This is (x-y) V_g. Clearing (9) of fractions and dividing by P^2 we obtain

$$V_g\left(x - y - \frac{2p}{P} - \frac{px}{P} - \frac{py}{P}\right) = 2\ Sp\left(1 - \frac{p^2}{P^2}\right) \tag{10}$$

This expression can be simplified since the fractions

$$\frac{px}{P},\ \frac{py}{P},\ \frac{p^2}{P^2},\ x,\ y,\ \frac{p}{P}$$

are small. Consequently

$$(x - y)\ V_g = h\left(\frac{V_g}{P} + 0.785\ d^2\right) \tag{11}$$

The expression within the parentheses on the right hand side of the equation (11) is the calibration factor. When multiplied by h it gives the difference in the volumes of gas change occurrung in vessels A and B. It should be pointed out that data obtained by this method are not extremely accurate as neither of these volumes is corrected for standard conditions.

When used as a closed system. The most valuable application of the differential manometer is the determination of the absolute magnitudes of changes in volume of the gas in one vessel, the other serving as a compensating vessel in which no reaction occurs. Warburg (1930) has presented a derivation of the calibration factor that is sufficiently accurate for general use. This derivation assumes that the menisci are observed with the manometer in a vertical position. The following numerical quantities are concerned:

P = initial pressure in millimeters of the liquid in the capillary.
P_o = millimeters of the liquid in the capillary equalling a pressure of one atmosphere.
h = difference in height of the menisci in the limbs of the manometer.
h' = increase in pressure in the compensating vessel due to the production of gas in the experimental vessels (this will be a negative value if gas is absorbed in the experimental value).
p = increase in pressure in the experimental vessel (this will be a negative value if gas is absorbed).
A = area in square millimeters of the cross section of the capillary.
V_g = gas-space in cubic millimeters in the experimental vessel.
V'_g = gas-space in the compensating vessel.
V_f = volume of liquids in cubic millimeters in the experimental vessel.
V'_f = volume of liquids in the compensating vessel.

T = absolute temperature.

α = Bunsen absorption coefficient of the gas which is changing volume in the experimental vessel in terms of cubic millimeters of liquid.

α' = Bunsen absorption coefficient of the gas in the compensating vessel (this is air unless the vessel has been swept out with some other gas).

Unlike the single manometer the change in pressure, Δp, in the experimental vessel is not equal to h, because the pressure changes in the opposite direction in the compensating vessel and therefore influences the movement of the menisci. After an evolution of gas, the pressure in the experimental vessel is $P + \Delta p$. The volume of the gas space is obviously increased to the extent of the volume of the capillary over the distance, h, which is

$$A \frac{h}{2} \tag{12}$$

and the total volume is equal to the original volume plus this quantity which is

$$V_g + A \frac{h}{2} \tag{13}$$

When this volume is corrected for standard temperature and pressure it becomes

$$\left(V_g + A \frac{h}{2}\right) \left(\frac{P + p}{P_o}\right) \left(\frac{273}{T}\right) \tag{14}$$

The change in volume is the difference between the new volume and the original volume, both being corrected for standard temperature and pressure. This is given by the expression

$$\left(V_g + A \frac{h}{2}\right) \left(\frac{P + p}{P_o}\right) \left(\frac{273}{T}\right) - \left(V_g \frac{P}{P_o} \frac{273}{T}\right) \tag{15}$$

If the area of the capillary is less than 0.5 square millimeters, the indicated subtraction can be simplified. Then factoring for Δp we obtain

$$\Delta p \left(\frac{V_g \frac{273}{T}}{P_o} + \frac{A}{2} \frac{273}{T} \frac{p}{P_o}\right) \tag{16}$$

Since the initial pressure is very nearly one atmosphere, it is very nearly true that

$$\frac{P}{P_o} = 1 \tag{17}$$

This permits a further simplification which yields

$$\Delta p \left(\frac{V_g \frac{273}{T}}{P_o} + \frac{A}{2} \frac{273}{T}\right) \tag{18}$$

The amount of gas dissolved in the liquid in the vessel is changed by the change in pressure. This change in dissolved gas is

$$V_f \alpha \frac{p}{P_o} \tag{19}$$

The total change in the volume (x) of gas in the experimental vessel when Δp is small, is then

$$x = \Delta p \left(\frac{V_g \frac{273}{T} + V_f \alpha}{P_o} + \frac{A}{2} \frac{273}{T} \right) \tag{20}$$

It is now necessary to eliminate Δp from equation (20) with the aid of h, which can be done in the following manner. If the difference in the levels of the menisci is h, then the volume in cubic millimeters of gas forced into the compensating vessel is

$$A \frac{h}{2} \frac{P}{P_o} \frac{273}{T} \tag{21}$$

This volume of gas must equal the decrease in the gas space in the compensation vessel. Let h' equal the change in pressure and by analogy with (18) and (19)

$$A \frac{h}{2} \frac{P}{P_o} \frac{273}{T} = h' \left(\frac{\left(V_g - A \frac{h}{2} \right) \frac{273}{T} + V'_f \alpha'}{P_o} \right) \tag{22}$$

Solving for h'

$$h' = h \left(\frac{\frac{A}{2} \frac{273}{T} \frac{P}{P_o}}{\frac{\left(V'_g - A \frac{h}{2} \right) \frac{273}{T} + V'_f \alpha'}{P_o}} \right) \tag{23}$$

This expression can be simplified, since h' is a correction term, P nearly equals P_o, and $A \frac{h}{2}$ is very small as compared with V'_g. We then obtain

$$h' = h \left(\frac{\frac{A}{2} \frac{273}{T}}{\frac{V'_g \frac{273}{T} + V'_f \alpha'}{P_o}} \right) \tag{24}$$

The actual change in pressure, Δp, which occurs in the experimental vessel is

$$\Delta p = h + h' \tag{25}$$

Substituting the value for h' obtained in (24) and factoring for h, we obtain

$$\Delta p = h \left(1 + \frac{\frac{A}{2} \frac{273}{T}}{\frac{V'_g \frac{273}{T} + V'_f \alpha'}{P_o}} \right) \tag{26}$$

Substituting in (20) the value obtained for Δp we obtain

$$x = h\left[1\left(+\frac{\frac{A}{2}\frac{273}{T}}{\frac{V'_g 273}{T} + V'_f\alpha'}{P_o}\right)\left(\frac{V_g\frac{273}{T} + V_f\alpha}{P_o} + \frac{A}{2}\frac{273}{T}\right)\right] \qquad (27)$$

The terms enclosed in the brackets are the calibration constant. *This derivation considers only the instance of a positive pressure developing in the experimental vessel.* It is not necessary to recalculate in the instance of the development of a negative pressure. One needs only to keep in mind that when multiplied by h it gives the volume of evolved gas in the first instance and of absorbed gas in the second.

If the area of the cross section of the capillary be very small the expression $\frac{A}{2}$ approaches zero and the following simplification results

$$x = h\left(\frac{V_g\frac{273}{T} + V_f\alpha}{P_o}\right) \qquad (28)$$

In other words, the calibration factor in this extreme case becomes equal to that of the constant volume single manometer. The physical effect of very small capillaries on the diffusion of gas precludes the use of this limiting condition in laboratory practice because pressure equilibrium is attained slowly and erratically.

It will be noticed that the derivation of equation (27) involves a number of assumptions. The error which results is less than 2 percent and consequently may be disregarded in ordinary laboratory procedures. For the highest accuracy, however, it is necessary to use the calibration factor derived by Dixon (1934). This derivation includes a correction factor for the angle of tilt of the manometer. The numerical quantities involved are the same except for the addition of p which is the vapor pressure of water at temperature T and $\Delta P'$ which is the change in pressure in the compensation vessel.

The volume of gas in the gas space of the experimental vessel under standard conditions is

$$V_g\frac{273}{T}\frac{P-p}{P_o} \qquad (1)$$

The final volume of the gas space by analogy with (3) is

$$\left(V_g + A\frac{h}{2}\right)\frac{273}{T}\frac{P-p+\Delta P}{P_o} \qquad (29)$$

And by analogy with (2) the amount of gas dissolved in the liquid is

$$V_f\alpha\frac{P}{P_o} \qquad (30)$$

The amount of gas produced will be the final volume of gas in the gas space (29) less the amount originally present (1) plus the amount which the new pressure has forced into solution (30 in the liquid present. This is

$$x = \left(V_g + A\frac{h}{2}\right)\frac{273}{T}\frac{P-p+\Delta p}{P_o} - V_g\frac{273}{T}\frac{P-p}{P_o} + V_f\alpha\frac{\Delta P}{P_o} \tag{31}$$

and

$$x = \left(V_g\frac{273}{T}\frac{\Delta P}{P_o}\right) + \left(A\frac{h}{2}\frac{273}{T}\frac{P-p-\Delta p}{P_o}\right) + \left(V_f\alpha\frac{P}{P_o}\right) \tag{32}$$

Factoring for ΔP we have

$$x = P\left(\frac{V_g\frac{273}{T} + V_f\alpha}{P_o} + \frac{A}{2}\frac{273}{T}\frac{(P-p)\frac{h}{\Delta P} + h}{P_o}\right) \tag{33}$$

In the compensating vessel, the amount of gas produced is zero, hence by analogy with (33)

$$0 = \Delta P'\left(\frac{V'_g\frac{273}{T} + V'_f\alpha'}{P_o}\right) - \left(\frac{A}{2}\frac{273}{T}\frac{(P-p)\frac{h}{\Delta P'} + h}{P_o}\right) \tag{34}$$

and

$$\left(V'_g\frac{273}{T} + V'_f\alpha'\right) = \left(\frac{A}{2}\frac{273}{T}\frac{(P-p+\Delta P')\,h}{\Delta P'}\right) \tag{35}$$

solving for $\Delta P'$ and factoring for h

$$\Delta P' = h\left(\frac{\frac{A}{2}\frac{273}{T}(P-p+\Delta P')}{V'_g\frac{273}{T} + V'_f\alpha'}\right) \tag{36}$$

When the manometer is in a slanting position, h does not represent the pressure difference between the vessels. It is apparent that h cos θ is the height of the liquid that represents the pressure changes. Since ΔP is the increase in pressure in the experimental vessel and $\Delta P'$ is the increase in the compensating vessel, h cos θ is the difference in these pressures and

$$h\cos\theta = \Delta P - \Delta P' \tag{37}$$

The assembling of the final equation proceeds as follows. From (37)

$$\Delta P = h\cos\theta + \Delta P' \tag{38}$$

Substitute the value of $\Delta P'$ obtained in equation (36)

$$\Delta P = h\left(\cos\theta + \frac{\frac{A}{2}\frac{273}{T}(P-p+\Delta P')}{V'_g\frac{273}{T} + V'_f\alpha'}\right) \tag{39}$$

In equation (33) substitute the value of ΔP found in (39)

$$x = h\left(\cos\theta + \frac{\frac{A}{2}\frac{273}{T}(P-p+\Delta P')}{V'_g\frac{273}{T} + V'_f\alpha'}\right)\left(\frac{V_g\frac{273}{T}V_f\alpha}{P_o} + \frac{\frac{A}{2}\frac{273}{T}(P-p)\frac{h}{\Delta P}+h}{P_o}\right) \quad (40)$$

If the manometer is not tilted, $\cos\theta$ is equal to 1 and then equation (40) becomes

$$x = h\left(1 + \frac{\frac{A}{2}\frac{273}{T}(P-p+\Delta P')}{V'_g\frac{273}{T} + V'_f\alpha'}\right)\left(\frac{V_g\frac{273}{T} + V_f\alpha}{P_o} + \frac{\frac{A}{2}\frac{273}{T}(P-p)\frac{h}{\Delta P}+h}{P_o}\right) \quad (41)$$

This strictly correct, but cumbersome formula may be simplified as described by Dixon (1934) with no significant loss in accuracy if V_g is about 40,000 cubic millimeters, V_f about 3,000 cubic millimeters, A is less than 0.5 square millimeter, and the length of the manometer scale is less than 200 millimeters. In equation (40) the relative values of the four main terms are then as follows: The second is $\frac{1}{15}$ of the first, the fourth is $\frac{1}{15}$ of the third. Errors of 15 percent in the second and fourth terms produce an error of only one percent in the final calibration constant. Also Δp differs from h only by two or three percent so we can assume

$$\frac{h}{\Delta p} = 1$$

And h cannot exceed 200, therefore, h and P' are negligible as compared to P. Also $P = P_o$ to within 5 percent. If no liquids are present which are more volatile than water, and if the temperature is less than 50° C. then p is less than 10 percent of P and therefore we can see that $P_o = P - p$ with an error of less than one percent. In the second term, $V'f\ \alpha$ does not exceed 5 percent of V'_g even when the gas concerned is carbon dioxide. With these simplifications, equation (40) becomes

$$x = h\left(\cos\theta\ \frac{\frac{A}{2}\frac{P_o}{V'_g}}{}\ \frac{V_g\frac{273}{T} + V_f\alpha}{P_o} + \frac{A}{2}\frac{273}{T}\right) \quad (42)$$

It was pointed out in a previous section that the sensitivity of the differential manometer does not continue to increase as the vessels become smaller. When extreme sensitivity is desired it is advantageous to use very small vessels and to open the stop cock on the side of the compensatory vessel. The system then functions as a single manometer since one limb is open to the air. The calibration factor for the Warburg constant-volume single manometer may not be used since the volume of the experimental vessel does not remain constant. If the compensatory vessel is open to the air, the value of V'_g becomes infinitely large.

If an infinitely large value for V'_g is inserted into equation (27) the following expression results.

$$x = h\left(\frac{V_g \frac{273}{T} + V_f\alpha}{P_o} + \frac{A}{2}\frac{273}{T}\right) \tag{43}$$

If the substitution be made in equation (42) and the manometer is not tilted, the same expression is derived

$$x = h\left(\frac{V_g \frac{273}{T} + V_f\alpha}{P_o} + \frac{A}{2}\frac{273}{T}\right) \tag{43}$$

Equation (43) is the one usually used to calculate the calibration constant for routine measurements when the differential manometer is used as a closed system.

IV. Routine Technique

A. Cleanliness

1. Capillaries

All types of micro technique demand the utmost cleanliness of the apparatus, and this is especially true of micromanometric methods. Any deviation from perfection in this regard will destroy the usefulness and dependability of the procedure. There are many instances in which workers have become discouraged and finally abandoned the use of micromanometric methods as impractical because they did not properly stress the importance of this essential aspect of routine technique.

Because of the smallness of the capillary of the manometer, factors affecting surface tension assume great importance. Before the manometer is put in use, the capillary must be *thoroughly cleaned and dried.* The stopcocks should first be freed from grease by being washed in reagent quality benzol. Then hot nitric acid should be drawn through the capillary, alternately in each direction. The washing effect of the acid is increased if bubbles of air are sucked into the tube at intervals. About 100 milliliters of the acid should be drawn through the capillary and then an equal volume of hot distilled water. Air is then drawn through until the capillary is dry. This drying operation should continue for a much longer time than seems reasonably necessary.

When all parts of the manometer have been cleaned and dried, the liquid should be placed in the capillary by means of a pipette drawn out to a long, fine point. The extension should be long enough to reach down through the stopcock into the capillary of the tube. This usually requires the capillary tip of the pipette to be four or five inches in length. One should avoid wetting the non-capillary parts of the manometer with the liquid. The cleanliness of the capillary can be checked by running the liquid to each end of the manometer scale alternately and then

letting it fall quickly to the point of rest. Any tendency of the dyed kerosene to cling to the walls of the tube indicates that the capillary is not clean. *This test always should be made before beginning an experiment.*

2. Stopcocks

The stopcocks should be greased, only after the capillary has received its liquid. The only grease that the author has found uniformly satisfactory is Merck's anhydrous lanolin. It should be in the clear, straw colored, vasoline-like form. Occasionally a whitish preparation is obtained which is too sticky to be satisfactory. The grease should be applied near the center of the shaft and then the shaft should be gently forced into place and slowly turned in one direction until the grease spreads over its entire surface. The grease should not be smeared on the entire shaft before it is put in place as this is likely to enclose droplets of air. The shaft of the stopcock should be free from any streaking when it is turned and the perforation of the plug should cut perfectly clean, leaving no streaks in its wake. Constant observation is necessary to make sure that such streaking has not occurred. Lanolin absorbs water and oxygen and gradually becomes sticky. When this occurs, the perforation will not cut clean when the shaft is turned. It is usually necessary to clean the stopcocks with benzol each week. The seating of a freshly greased stopcock shaft always forces a little grease into the manometer tube. This can be removed by a pipe-stem cleaner that has been washed free of lint with kerosene.

3. Vessels

The vessels should be cleaned thoroughly at the end of each experiment. Excess grease should be wiped from the ground joint with clean cheesecloth wrapped on the end of a glass rod. The joint is then wiped with clean bits of cloth wetted with reagent quality benzol, until the ground surface shows no streaks on drying. The content of the vessel may then be washed out, first with water and then with cleaning solution if desired, and again with water, and finally with anhydrous ether. To do this before the ground joint surface had been cleaned makes the grease sticky and more difficult to remove. The cleaned vessels should be stored, lying on their side, in a dust-free cabinet until again used.

The ground joints of the arms of the manometer also must be cleaned with benzol each time the manometer is used.

4. Sterile Technique

If it is desired to operate with sterile technique, it is sufficient to wash the vessels finally with anhydrous acetone and store them in a dust-free cabinet suspended in a frame with their opening downward. They may be used without further treatment, excepting a quick flaming of the opening before sterile liquids are pipetted into the reaction chamber.

B. PIPETTING

The transference of the small quantities of liquids involved in micro-manometric methods requires the same care as the finest volu-

metric procedures of analytical chemistry. High grade pipettes are calibrated by the manufacturer to deliver the stated volume only if the liquid runs out of its own weight. The liquid should never be blown out, for the rate of flow determines the amount of the liquid which adheres to the walls of the pipette, and the drop that hangs in the tip is not included in the calibrated volume. Very small quantities can be delivered from a 1-milliliter graduated pipette whose tip has been drawn out into a capillary with an outside diameter of about one millimeter. Liquids can be conveniently placed in the side arms of the vessels by capillary pipettes with curved tips. Experience will show what this curve must be to fit different types of vessels. In order to prevent errors due to the liquid which drains down the outside of the pipette, a slight excess of the liquid should be drawn in and the outside wiped dry with a clean cloth. Then the excess liquid is allowed to run out, and the tip cleared of the hanging drop of liquid by touching it to a piece of filter paper. After the proper delivery has been made, the tip is again cleared of the hanging drop by touching it to the side of the vessel. Practice with properly constructed pipettes will enable the operator to deliver aliquots as small as 0.1 milliliter with great precision. Pipettes with capillary tips must be cleaned and dried after use in order to prevent the sealing of the openings. It is superfluous perhaps to add that the walls of pipettes must be perfectly clean in order to prevent variable amounts of liquid from adhering to them.

C. ALKALI

The concentration of the sodium or potassium hydroxide used to absorb carbon dioxide should be about 2 normal, although this concentration is not critical. Since carbon dioxide absorption is limited to the exposed surface film, it is useless to add more alkali once the maximum area of its exposed surface has been reached in the inset or the side arm. Many types of reactions eliminate carbon dioxide so rapidly that the rate of its diffusion into the limited surface of the alkali affects the pressure changes observed by the manometer. This is especially true of yeast and bacteria, and in general those reactions carried out at 37° C. In such instances, the absorbing surface is increased by inserting a tiny roll of filter paper made from squares of about one inch or less into the inset, and then wetting with the alkali. It should not be necessary to use more than 0.3 to 0.4 milliliter of the alkali solution in any case. The rim of the inset or side arm containing the alkali must be kept dry in order to prevent creeping of the liquid. The mixing of the alkali with the experimental liquid by creeping is a frequent cause of unsuccessful experiments.

D. MENISCUS

The highest accuracy in the observation of gaseous changes in liquids demands that the meniscus be read while the manometer is in motion. The instant that the motion ceases, the gas equilibrium between the liquid and the atmosphere above it is disturbed. The motion of the constant volume single manometers must be stopped in order to adjust the levelling device, and that is one of the disadvantages of their use. The ability of accurately reading the meniscus while it is in motion is

acquired only with practice. Observations on tissue which is not in a liquid do not require that the manometer be rocked.

The comparatively high surface tension effects in small capillaries cause a certain amount of stretching of the surface film of the liquid forming the meniscus. For this reason, the first movement observed is to be disregarded. The first reading of the meniscus should not be recorded until it has been in motion under the conditions of the experiment for at least 10 minutes.

The movement of the meniscus in each limb of the manometer is recorded separately and is based on its position when the first reading of the experiment was made. If the capillary is sufficiently even in bore, the movement of each meniscus from the position of the first reading will be equal to within ± 0.5 millimeter. The highest accuracy demands that the total movement of each meniscus be the same to within this value.

Experience has shown that pressure changes can be observed most accurately if the menisci move at a rate of two to five millimeters per 10 minutes. Experienced operators can read the position of the meniscus to ± 0.1 millimeter even though the manometer is in motion. The reading is facilitated if the operator holds an electric light, shaded from the eyes, just below his line of vision. The kerosene stained with Sudan III in the capillary then gives a sharply defined meniscus against the millimeter scale mounted behind the capillary. It should be remembered that the heat from the light will quickly affect the size of the bore of the manometer as well as the volume of the gas within it.

E. SAMPLE

Whenever possible, the size of the sample and the size of the vessel should be chosen that the menisci will move from two to five millimeters per 10 minutes.

F. RECORDING OF DATA

As a great many readings are made in the course of a single experiment, it is convenient to record the data on mimeographed forms. An electric clock with a large second hand should be within easy vision of the operator. As the second hand marks the indicated time, the manometers are read always in the same order and at the same speed. This makes it possible for the time recorded to apply to all the manometers. It is not possible, even for a skilled operator, to read more than six manometers without recording a new time if highly precise data are desired. There is usually sufficient time between readings to allow the operator to apply the calibration factors to each reading and to record it on graph paper. Whenever possible, the graph should be drawn point by point as the data are obtained, since the shape of the curves gives a picture of the proper functioning of the manometer. The data should be graphed on the same type of paper as that used for the manometer scale.

G. CHECKING OF TECHNIQUE

The apparatus and procedure should be carefully checked before the collection of research data is attempted. The behavior of the manometers first should be observed when they contain only non-

reacting liquids. This will enable the operator to detect the effect of temperature and the length of time that is necessary for any given set of vessels to come to temperature equilibrium. Vessels of a volume of 10 milliliters or less will come to temperature equilibrium in 10 to 20 minutes. Larger vessels may require 30 minutes or more.

The most important phase of the preliminary checking is the observation of the results obtained with duplicate samples. The variations observed with duplicate samples include the errors due to the apparatus, and those in sampling, as well as those of the technique of the operator. *Not until the magnitude of these has been determined by repeated experiments, should the collection of research data be attempted.*

H. SUMMARY

The inherent accuracy of micro-manometric methods is very great, and numerous physiological processes may be advantageously studied by these procedures. But as in the application of any micro method, there can be no such thing as variation in degrees of technical accuracy, for the observations are either *accurate* or *worthless*. The equipment itself must be mechanically perfect. The calibration factors must be determined by means of carefully obtained measurements of the area of the cross section of the capillary, the volumes of gas and liquid in the vessels. The skill of the operator depends on his maintaining clean capillaries, perfect greasing of stopcocks, accurate pipetting, timing, and reading of the menisci. It should be remembered that not all persons have an equal facility for acquiring technical skill. Some will obtain experimental data beautiful in their accuracy and dependability. Others can never succeed, regardless of all effort expended toward acquiring the necessary manual dexterity.

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Egg Culture Studies of Fungi[1]

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In a series of studies undertaken to obtain information in order to establish a screen testing program, with fungicidal drugs, embryonated eggs were selected. It was observed that some of the pathogenic fungi were so lethal for the eggs that no time was allowed for the action of the drug before the egg died. In an attempt to determine dilutions of the inoculum of the fungus that would allow time for drug action and yet develop a degree of pathogenicity for the egg, five species of fungi were used. In each instance, .1 cc of a 24 hour Sabouraud's acid broth culture was used as well as .1 cc of a broth culture diluted to 10^{-6} and .1 cc diluted to 10^{-12}. Four eggs were inoculated from each dilution. Parallel inoculations were made with three of the species using inoculum from eggs recently killed by inoculation with that particular species. In the case of *Monilia tropicalis*, plates were run with the inoculum from an egg yolk sac that produced five colonies per .1 cc of yolk sac material diluted to 10^{-12} and from Sabouraud's acid broth that produced approximately 25,000 organisms per .1 cc for the 10^{-12} dilution. The results of the inoculations are indicated on Chart 1.

Chart 1. A series of titrations of fungus cultures was inoculated into embryonated eggs. One series was inoculated with titrations of cultures taken from the yolk sacs of eggs which had just died from the fungus. The other series were inoculated with titrations of twenty-four hour cultures in Sabouraud's acid broth. In each case .2 cc. of inoculum was used per egg. Four eggs were inoculated with each dilution, the dilutions being used were 10^{-0}, 10^{-6} and 10^{-12}. Eggs living eleven days were discarded.

Sabouraud's broth culture inoculation of Candida tropicalis		Sabouraud's broth culture inoculation of Candida albicans		Sabouraud's broth culture inoculation of Candida Krusei	
10^{-0}	xxxxxxxxxxx	10^{-0}	xxxxxx	10^{-0}	xxx
10^{-0}	x	10^{-0}	xxxxx	10^{-0}	xxx
10^{-0}	x	10^{-0}	xxxx	10^{-0}	xxx
10^{-0}	x	10^{-0}	xxxx	10^{-0}	xxx
10^{-6}	xxxxxxxxxxx	10^{-6}	xxxxxxxxxxx	10^{-6}	xxxxxxxxxxx
10^{-6}	xxxxxxxxxxx	10^{-6}	xxxxxxxxxxx	10^{-6}	xxxxxxxxxxx
10^{-6}	xxxxxxxxxxx	10^{-6}	xxxxxxxxxxx	10^{-6}	xxxxx
10^{-6}	xxxxxxxxxxx	10^{-6}	xxxxxxxxxxx	10^{-6}	xxx
10^{-12}	xxxxxxxxxxx	10^{-12}	xxxxxxxxxxx	10^{-12}	xxxxxxxxxxx
10^{-12}	xxxxxxxxxxx	10^{-12}	xxxxxxxxxxx	10^{-12}	xxxxxxxxxxx
10^{-12}	xxxxxxxxxxx	10^{-12}	xxxxxxxxxxx	10^{-12}	xxxxxxxxxxx
10^{-12}	xxxxxxxxxxx	10^{-12}	xxxxxxxxxxx	10^{-12}	xxxxxxxxxxx

[1]Supported by a grant from the Dr. Leonard H. and Louis Weissman Medical Research Foundation.

Chart 1—(*Continued*)

Sabouraud's broth culture inoculation of Aspergillus sp.		Sabouraud's broth culture inoculation of Cephalosporium sp.	
10^{-0}	xxx	10^{-0}	xxxxxxxxxxx
10^{-0}	xxx	10^{-0}	xxxxxxxxxxx
10^{-0}	xx	10^{-0}	xxxxxxxxxxx
10^{-0}	xx	10^{-0}	xxxxxxxxxx
10^{-6}	xxx	10^{-6}	xxxxxxxxxxx
10^{-6}	xxx	10^{-6}	xxxxxxxxxxx
10^{-6}	xxx	10^{-6}	xxxxxxxxxxx
10^{-6}	xxx	10^{-6}	xxxxxxxxxxx
10^{-12}	xxxxx	10^{-12}	xxxxxxxxxxx
10^{-12}	xxxx	10^{-12}	xxxxxxxxxxx
10^{-12}	xxxx	10^{-12}	xxxxxxxxxxx
10^{-12}	xxx	10^{-12}	xxxxxxxxxxx

Yolk sac content inoculations of Candida tropicalis		Yolk sac content inoculations of Candida albicans		Yolk sac content inoculations of Aspergillus sp.	
10^{-0}	xxx	10^{-0}	xxxxxxxxxxx	10^{-0}	xxxxx
10^{-0}	xxx	10^{-0}	xxxxxxxxxxx	10^{-0}	xxx
10^{-0}	xx	10^{-0}	xxxxxx	10^{-0}	xxx
10^{-0}	xx	10^{-0}	xx	10^{-0}	xxx
10^{-6}	xxxxxxxxxxx	10^{-6}	xxxxxxxxxxx	10^{-6}	xxxxxxxxxxx
10^{-6}	xxxxx	10^{-6}	xxxxxxxxxxx	10^{-6}	xxxxxxxxxxx
10^{-6}	xxx	10^{-6}	xxxxxxxxxxx	10^{-6}	xxxxxxxxxxx
10^{-6}	xxx	10^{-6}	xxxxxxxxxxx	10^{-6}	xxxxxxxxxxx
10^{-12}	xxxxxxxxxxx	10^{-12}	xxxxxxxxxxx	10^{-12}	xxxxxxxxxxx
10^{-12}	xxxxxxxxxxx	10^{-12}	xxxxxxxxxxx	10^{-12}	xxxxxxxxxxx
10^{-12}	xxxxxxxx	10^{-12}	xxxxxxxxx	10^{-12}	xxxxxxxxxxx
10^{-12}	xxxxx	10^{-12}	xx	10^{-12}	x

The 10^{-0} dilutions of the culture of *Monilia albicans* appear to be an ideal concentration. Dilutions between 10^{-0} and 10^{-6} are indicated in the case of *Monilia krusei*. A dilution of 10^{-12} was most satisfactory for an Aspergillus species. The results of the culture inoculations of *Monilia tropicalis* are inexplicable by the authors. In the case of Cephalosporium, the inoculum was not sufficiently concentrated for best results. The results obtained by direct inoculation from infected eggs appeared the most satisfactory for Aspergillus at 10^{-0} concentration. In the case of the two Monilias, spotted killing by *albicans* was observed. The concentration for the MLD for *Monilia tropicalis* lies somewhere between 10^{-0} and 10^{-6}.

An additional series of dilutions was made using normal saline for the titrations. The original inoculum was taken in each case from 24 hour old Sabouraud's acid broth using .1 cc injections. Injections of 10^{-0}, 10^{-2}, 10^{-4} and 10^{-6} were used. Seven fungi were used for the tests. The results of the inoculations are indicated in Chart 2.

CHART 2. A series of titrations of fungus cultures was inoculated into embryonated eggs. For each fungus two eggs were inoculated with .1 cc. injections of Sabouraud's acid broth twenty-four hour cultures, in 10^{-0}, 10^{-2}, 10^{-4} and 10^{-6} dilutions. Living eggs were discarded on the eighth day. In the case of four species of Monilia cultures were made from the eggs when they died or

were discarded, a plus or a minus indicates the presence or absence of the Monilia in stabs made from the broth. An "x" indicates a day that the particular egg lived.

Cephalosporium sp.		Aspergillus sp.		Candida albicans (PM strain)	
10^{-0}	xxxxxxxx	10^{-0}	xxxxx	10^{-0}	xx
10^{-0}	xxxxxx	10^{-0}	xxxx	10^{-0}	x
10^{-2}	xxxxxxxx	10^{-2}	xxxxxxxx	10^{-2}	xx
10^{-2}	xxxxxxxx	10^{-2}	xxxxxxxx	10^{-2}	xx
10^{-4}	xxxxxxxx	10^{-4}	xxxxxxxx	10^{-4}	xx
10^{-4}	xxxxxxxx	10^{-4}	xxxxxxxx	10^{-4}	xx
10^{-6}	xxxxxxxx	10^{-6}	xxxxxxxx	10^{-6}	xx
10^{-6}	xxxxxxxx	10^{-6}	xxxxxxxx	10^{-6}	xx

Candida tropicalis		Candida Krusei		Candida albicans	
10^{-0}	xxxxxxxx+	10^{-0}	xxx+	10^{-0}	xxxx+
10^{-0}	xxxxxxxx+	10^{-0}	xxx+	10^{-0}	xxxx+
10^{-0}	xxxxx+	10^{-0}	xxx+	10^{-0}	xxxx+
10^{-2}	xxxxxx+	10^{-2}	xxxxxxx+	10^{-2}	xxxxxxxx+
10^{-2}	xxxx+	10^{-2}	xxxxxx+	10^{-2}	xxx+
10^{-4}	xxxxxx+	10^{-4}	xxxxxxx+	10^{-4}	xxxxxxxx+
10^{-4}	xxxxx+	10^{-4}	xxxxxx+	10^{-4}	xxxx+
10^{-6}	xxxxxxxx−	10^{-6}	xxxxxxxx+	10^{-6}	xxxxxxxx−
10^{-6}	xxxxxxxx−	10^{-6}	xxxxxxxx+	10^{-6}	xxxxxxxx−

Candida pseudotropicalis	
10^{-0}	xxxxxxxx+
10^{-0}	xxxxx+
10^{-2}	xxxxxx+
10^{-2}	xxxx+
10^{-4}	xxxxxxxx+
10^{-4}	xxxxxx+
10^{-6}	xxxxxxxx+
10^{-6}	xxxxx+

The results indicate that with the dilution method an inoculum may be established that will permit the egg to survive long enough for the activity of the drug to be observed. Once this is established for a particular inoculum methods for maintaining the original culture for continued use without reworking the MLD each time, will have to be established.

Studies on Monilia[1]

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The usual procedure for the determination of monilias is to culture them by the stab method on cornmeal agar plus fermentation reactions with sugars plus cultural characteristics (cf. page 142, Conant, Norman F., *et al.* Manual of Clinical Mycology. W. B. Saunders Co. Phila. 1949). Those yeasts which do not produce mycelia when stabbed on cornmeal agar by this routine are considered as being non-pathogenic *Saccharomyces.*

After some studies of the growth characteristics of various species of monilias in embryonated eggs it was decided to culture some of the "non-pathogenic yeasts" in the embryonated eggs for comparison. For this, routine cultures made from vaginal smears from the clinic were used. Seven cultures were selected at random, all of them routinely stabbed on cornmeal agar without producing mycelia and determined as *Saccharomyces.* In each case material from an isolated colony was used, the material being placed in Sabouraud's acid broth (pH 4) and incubated at 37° C for twenty four hours. Then .1 cc of the culture was inoculated into the yolk sac of one seven day embryonated egg. When an egg died, .1 cc of inoculum was obtained from the yolk sac of the dead egg for inoculation into another embryonated egg between seven and fourteen days old. Additional inoculum was placed in Sabouraud's acid broth and after twenty four hours stabs were made from this into cornmeal agar. Table 1 indicates the results of these inoculations.

Table 1. *Chart showing the results of inoculations of seven different "non-pathogenic yeasts" originally cultured from vaginal smears into embryonated eggs.* An "x" indicates one day of survival of the embryo. A gap between the "x's" indicates the death of an embryo and its reinoculation into another egg. A minus indicates failure to produce mycelia with cornmeal agar stabs, while a plus indicates that mycelia were produced. After each series is indicated the fermentation reactions of each yeast; a double plus indicates acid and gas, a single plus indicates acid production without gas, and a negative indicates production of neither acid nor gas. The sugars used were dextrose, sucrose, lactose and maltose, reading from left to right.

220 − xxxx + xxxx + xxx − xxx −	+	−	−	++
222 − xx + xxxxxxxxxx +	+	−	−	+
223 − xx + xxxx + xxx + xxx − xx −	+	−	−	++
224 − xx − xxx + xxxxxxx +	++	−	−	++
225 − xx − xxxxxxxxxx −	+	−	−	++
227 − xxxxx + xxxxx + xx −	++	−	−	++
232 − xxxxxxxxxx + xxx −	−	−	−	−

[1]Supported by a grant from the Dr. Leonard H. and Louis Weissman Medical Research Foundation.

It will be noted that after one passage in the egg five of the seven yeasts produced mycelia, and that another one produced mycelia after passage through two eggs. The mycelia were not abundant, and while consistent for each culture, there were three distinctive types of mycelia among the six cultures. The fermentations appear to be those of *Candida stellatoidea* Jones and Martin, but the three types of mycelia seem to indicate that more than one species of fungus are involved in the series. The authors are inclined to believe that monilial infections are somewhat more common than is usually believed, and that some of the "non-pathogenic" yeasts obtained in undiagnosed generalized infections may be worthy of additional study.

A Simple Technique for Collecting Fungus Specimens from Infected Surfaces

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The difficulties of collecting and preparing fungus specimens for direct examination from skin and of making adequate transfer to culture media are well known. If scarifying is done it is difficult to gather the material which has been loosened. Those methods which the authors have previously tried are less than satisfactory.

In those cases, in which the fungus or its spores are superficial, acetate film adhesive tape[1] can be used to pick up samples[2]. In cases where there is very little loose material, the area can be scarified before using the tape. A convenient length (5–15 cm) of tape 1 inch wide is cut off, and, holding the two ends, the tape is pressed against the infected area. Pulling the tape sidewise while applying pressure aids in dislodging scales. Since the acetate film is transparent the amount collected can usually be seen using a hand lens if necessary. A glass cut to a size of 6″ x 8″ acts as a satisfactory method of preparing the tape for ready access. The strips of tape are cut about 6″ in length, applied to a clean plate, and disinfected with alcohol. The ends of the tape are raised from the plate and a small cardboard tab is attached to each end. This allows much easier handling of the tape.[3]

For culturing, a portion of tape with sample can be cut off and dropped into Sabouraud's broth, another portion placed on a clean flamed slide for transporting to the laboratory where it is removed and placed adhesive side down on a suitable agar plate. Another piece of tape with sample is placed on a slide, and in the laboratory the film is peeled back for applying hydroxide or suitable stain with the film serving as coverslip. The acetate film, but not the usual transparent tapes, resists the hydroxide and is suitable for oil immersion examination.

The tape in the roll has been found not to have fungus spores and the contamination hazards are those from brief air exposure of the adhesive side, handling contamination at the edges and on the non-adhesive side. Wiping the roll with alcohol and sterile cotton and trimming off the sides and ends of the film will reduce the chances of contamination to a minimum. Surface cleansing of the fungus infected area will in most cases reduce the amount of fungus collected and is usually contraindicated.

Material from human cases and from plant leaves has been collected by the above method. The technique can be adapted for taking samples from interior body surfaces as the ingenuity and manipulative skill of the operator permit since the adhesive will take some material from moist surfaces.

The above outlined technique has also been applied by Barkley[4] to obtaining study material from leaves and stems in the study of pathogenic plant fungi.

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[1]Acetate film tape No. 800, Minnesota Mining and Mfg. Co., St. Paul 6, Minn.

[2]Edwards, R. W.: Scotch tape-slides for rapid identification of pathogenic fungi. Lab. Dig. *15:* 8–9, 1951.

[3]Pollard, A. L., University of Tennessee, personal communication.

[4]Barkley, Fred A., Hektoen Institute, personal communication.

Ethyl Vanillate* Studies on Embryonated Eggs. I.

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The comparative results of a study on controlling diseases of different groups of organisms in embryonated eggs by some chemical were thought to be desirable. For this study a chemical was desired regarding which extensive comparative studies had not been made, but which promised some indication of effectiveness. Ethyl vanillate was chosen, as its toxicity in humans is rather well known[1], it has been used in the treatment of a disease (histoplasmosis)[1], its structure is well known[2,3] and it is used as a food preservative.[4]

Toxicity tests were run on ethyl vanillate in embryonated eggs and various groups of pathogenic organisms were "screen tested" with ethyl vanillate in embryonated eggs. The present note concerns the toxicity tests, while the results of the testing on pathogens will be the subject of further notes.

Since ethyl vanillate is only slightly soluble in water, the higher concentrations of one series of solutions were made with ethyl alcohol for injection into the eggs. (From the results it will be seen that dilutions of alcohol higher then 15 percent were toxic, and only by diluting the higher concentrations with water and then introducing a sufficient quantity to give a desired quantity of ethyl vanillate could some of the testing be done.) The series of solutions with ethyl alcohol were as follows:

"O"	.5	g.	ethyl	vanillate	in	1	cc.	of	90	percent ethyl alcohol
"A"	.05	g.	"	"	"	"	"		50	" " "
"B"	.005	g.	"	"	"	"	"		15	" " "
"C"	.0005	g.	"	"	"	"	"		1.5	" " "
"D"	.00005	g.	"	"	"	"	"		.15	" " "

Since ethyl vanillate is readily soluble in peanut oil several dilutions were tested, as follows:

"E"	1	g.	ethyl	vanillate	per	1	cc. peanut oil
"F"	.2	g.	"	"	"	"	" "
"G"	.1	g.	"	"	"	"	" "

For comparison with embryonated eggs a series of mice was tested with ethyl vanillate solutions. (1) Three mice were injected in the muscles of the back with .2 cc. of solution A. After two days they

*Supplied by The Institute of Paper Chemistry, Appleton, Wis. Supported by a grant from the Dr. Leonard H. and Louis Weissman Medical Research Foundation.

[1]Christie, Amos, James G. Middleton, J. Cyril Peterson and David L. McVickar. Treatment of disseminated Histoplasmosis with Ethyl Vanillate. Pediatrics 7: 7–18. 1951.

[2]Pearl, I. A. Reactions of Vanillin and its Derived Compounds. I. J. Am. Chem. Soc. *68*: 429. 1946.

[3]Pearl, I. A. Reactions of Vanillin and its Derived Compounds. IV. J. Am. Chem. Soc. 2180. 1946.

[4]Pearl, I. A., and J. F. McCoy. Vanillic Acid Esters as Food Preservatives. Food Industries *17*: 1458. 1945.

showed no toxic effects and were discarded. (2) Three mice were injected interperitoneally with .2 cc. of solution A. After two days they showed no toxic effects and were discarded. (3) Three mice were injected in the back muscles with .1 cc. of solution O. After five days they showed no toxic effects and were discarded. (4) Three mice were injected interperitoneally with .1 cc. of solution O. After five days they showed no toxic effects and were discarded. (5) Two mice were injected interperitoneally with .2 cc. of solution A, followed by two injections of .1 cc. of solution A, twenty-four hours apart. After five days they showed no toxic effect and were discarded. (6) Two mice were injected interperitoneally with .1 cc. of solution O, followed by two injections of .1 cc. of solution O, twenty four hours apart. After five days they showed no taxic effect and were discarded. (7) Three mice were injected subscutaneously with .3 cc. of solution E. Two of the mice showed toxic effects almost immediately and died in about 15 minutes. The third mouse behaved drunkenly for several hours, but gradually recovered and was discarded on the fifth day showing no further effects of toxicity.

A series of three eggs each was injected with various peanut oil dilutions, with those living through 12 days being discarded. The results may be seen in Table 1. From these it appears that peanut oil is not toxic to the eggs and that dilutions of ethyl vanillate less than .2 g. per cc in dosages of .1 cc. are not toxic, while higher concentrations are definitely toxic.

A series of seven day embryonated eggs was inoculated with .1 cc. of solution. Those living through 12 days were discarded. The yolk sac and amniotic inoculations were repeated for the O and A solutions, diluting the injections with four parts of water. The results of this series of injections may be seen in Table 2. It would appear from the results that there is no particular difference as to the route of entrance of the chemical, but that the toxicity of the higher concentrations is largely due to the ethyl alcohol, which when diluted cause little toxicity.

A series of fourteen day embryonated eggs was inoculated with .1 cc. of solution. Those living through six days were discarded. The results of these injections will be seen in Table 3. It will be noted that the fourteen day eggs are much more resistant to the effect of the alcohol and that the dropping of the membrane is more deleterious on them.

From this series of injections it will be seen that ethyl vanillate is toxic on embryonated eggs in its higher concentrations, but .1 cc. injections of concentrations of .2 g. per cc. are not particularly so.

TABLE 1. A series of three eggs each was injected with various peanut oil dilutions of ethyl vanillate. Each "x" represents one day that the egg survived. Eggs living twelve days were discarded.

Eggs without peanut oil nor ethyl vanillate.	xxxxxxxxxxx xxxxxxxxxxx x
.1 cc. per egg of peanut oil without ethyl vanillate.	xxxxxxxxxxx xxxxxxxxxxx xxxxxxxxx

Table 1—(*Continued*)

.1 cc. per egg of peanut oil solution "G"	xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxx
.1 cc. per egg of peanut oil solution "F".	xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxx
.1 cc. per egg of peanut oil solution "E".	xxxx xxx xxx

TABLE 2. A series of three eggs each was injected with various ethyl alcohol dilutions of ethyl vanillate by three types of inoculations. The eggs in this series were seven day embryonated eggs. Those living twelve days were discarded. .1 cc. of each solution was injected into each egg, except in the case of the A* and O* eggs where the .1 cc. was injected with .4 cc. water.

Solution	Yolk sac inoculations	Amniotic inoculations	Dropped membrane inoculations
O	x	x	
O	x	x	
O	x	x	
O*	xxxxxxxxxxxx	xxxxxxxxxxxx	
O*	xxxxxxxxxxxx	xxxxxxxxxxxx	
O*	xx	xxxx	
A	xx	x	xxxxxxxxxxxx
A	xx	x	xxxx
A	x	x	x
A*	xxxxxxxxxxxx	xxxxxxxxxxxx	
A*	xxxxxxxxxxxx	xxxxxxxxxxxx	
A*	xxxxxx	xxxxxxxxxxxx	
B	xxxxxxxxxxxx	xxxxxxxxxxxx	
B	xxxxxxxxxxxx	xx	
B	xx	x	
C	xxxxxxxxxxxx	xxxxxxxxxxxx	xxxxxxxxxxxx
C	xxxxxxxxxxxx	xxxxxxxxxxxx	xxxxxxxxxxxx
C	xxx	x	xxx
D	xxxxxxxxxxxx	xxxxxxxxxxxx	
D	xxxxxxxxxxxx	xxxxxxxxxxxx	
D	xxxxxxxxxxxx	xxxxxxxxxxx	

TABLE 3. A series of three eggs each was injected with various ethyl alcohol dilutions of ethyl vanillate by three types of inoculations. The eggs in this series were fourteen day embryonated eggs. Those living six days were discarded. .1 cc. of each solution was injected into each egg.

Solution	Yolk sac inoculations	Amniotic inoculations	Dropped membrane inoculations
A	xxxxxx	xxxxxx	xxxxxx
A	xxxxxx	xxxxxx	x
A	xxxxxx	xxx	x
B	xxxxxx	xxxxxx	
B	xx	xxxxxx	
B	x	x	
C	xxxxxx	xxxxxx	xxxxxx
C	xxxxxx	xxxxxx	xxxxxx
C	xxxxxx	xxxxx	x
D	xxxxxx	xxxxxx	
D	xxxxxx	xxxxxx	
D	xxxxxx	xxxxxx	

Ethyl Vanillate* Studies on Embryonated Eggs. II.

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A small group of viruses and rickettsias was used in a series of inoculations of ethyl vanillate to obtain an indication of the latter's activity against them. The dilutions of ethyl vanillate used were:

"O"	.5.5	g. of ethyl vanillate per cc. of	95	percent ethyl alcohol
"A"	.05	g.	"	"	"	"	"	50	"	"	"
"B"	.005	g.	"	"	"	"	"	15	"	"	"
"C"	.0005	g.	"	"	"	"	"	1.5	"	"	"
"D"	.00005	g.	"	"	"	"	"	.15	"	"	"

The first virus tested was vaccinia. Three eggs were tested by the drop membrane method with .2 cc. of a suspension of vaccinia virus and .1 cc of the ethyl vanillate solution. After three days the eggs were opened and observed for presence of activity of the virus. Of three eggs inoculated with solution "A" and the virus, one had died previously and two were alive and without signs of the activity of vaccinia. Of the three eggs inoculated with solution "B" and the virus, one had died previously and two were alive and showed signs of the activity of vaccinia. Of the three control eggs, inoculated with the virus but not with ethyl vanillate solution, all three were alive and showed definite signs of activity of vaccinia. This was repeated using two eggs with "O" solution, two with "A" solution, and two controls. The eggs with solution "O" were both negative to signs of activity of vaccinia, one of the solution "A" eggs was positive and one negative to the activity of vaccinia, and the controls were both positive. From these results it would seem that ethyl vanillate has some effect on the growth of the vaccinia virus.

The second virus tested was the virus of Newcastle disease. Three eggs each were inoculated with 10^{-7} and 10^{-10} suspensions of the Newcastle virus. A, B, C, and D dilutions of ethyl vanillate were used with .1 cc. of the Newcastle suspension and .1 cc. of the solution of ethyl vanillate. At the end of three days the eggs which were dead were harvested and given the hemaglutination test for the presence of the virus. The results are given in Chart 1. A second series of inoculations

Chart 1. Showing the effects of hemaglutination tests on the dead eggs of a series inoculated with various dilutions of ethyl vanillate and with various titrations of Newcastle virus.

AN^{-7} − − −	AN^{-10} −	
BN^{-7} ± − −	BN^{-10} ± + −	
CN^{-7} + −	CN^{-10} ± ± +	
DN^{-7} + −	DN^{-10} ± ±	
N^{-7} + −	N^{-10} + + +	N^{-18} + + +

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were made using a suspension of Newcastle of 10^{-14}, and dilutions O and A of ethyl vanillate. Three eggs were inoculated with each dilution of ethyl vanillate and three control eggs were inoculated with only the Newcastle suspension. The results are given in Chart 2. It

CHART 2. Showing the effects on the death rate of eggs inoculated with Newcastle virus and dilutions of ethyl vanillate. The living eggs were discarded on the thirteenth day. Each egg was inoculated with .1 cc. of a suspension of 10^{-14} dilution of Newcastle virus, and those indicated as receiving ethyl vanillate with .1 cc. of the diluted ethyl vanillate. An "x" indicates one day that the egg lived.

ON^{-14}	xxxxxxxxxxxx	AN^{-14}	xxxxxxxxxxxx	N^{-14}	xxxx
ON^{-14}	xxxx	AN^{-14}	xxxxxxxxxxxx	N^{-14}	xxxx
ON^{-14}	xx	AN^{-14}	xxxxxxxxxxxx	N^{-14}	xxx

seems probable that some of the deaths indicated in Chart 2 for the highest concentration are in reality due to toxicity to the ethyl alcohol. From these results it would seem that ethyl vanillate definitely has some control over the growth of Newcastle virus.

The third virus tested was that of influenza (common strain). This was given as .1 cc. allantoic inoculations accompanied in all but the controls with .1 cc. of the diluted ethyl vanillate. The A, B, C, and D dilutions of ethyl vanillate were tested. A second series of eggs using the O and A dilutions were tested. At the end of three days the living eggs were tested by the hemaglutination test for the presence of the influenza virus. The results are shown in Chart 3. From these

CHART 3. Chart showing the results of hemaglutination tests on eggs inoculated with the common strain of influenza. Three eggs for each dilution of the ethyl vanillate dilution and three controls were inoculated with .1 cc. of suspension of influenza while an additional .1 cc. of the dilution of ethyl vanillate was added to each of the eggs except the controls. At the end of three days the living eggs were given the hemaglutination test with the results shown.

FA	− − −	FO	− −
FB	− + ±	FA	− − −
FC	− + ±		
FD	− − +		
F	+ + +	F	+ +

results it would appear that ethyl vanillate has some control over the persistance of the influenza virus in the embryonated egg.

The fourth virus tested was that of feline pneumonitis. Four each of ten day old embryonated eggs were inoculated with .1 cc. of a suspension of virus of feline pneumonitis and except for the controls with .1 cc. of the dilution of ethyl vanillate. The dilutions of ethyl vanillate used were the O, A, B, C, and D solutions. A second series of three eggs each was run on seven day embryonated eggs using dilutions O and A. The results of this experiment are shown in Chart 4. The excessive killing by the O dilution is probably due to the presence of

CHART 4. Chart showing the effects of several dilutions of ethyl vanillate on ten day embryonated eggs in controlling feline pneumonitis, four eggs being used for this for each dilution, and of ethyl vanillate on seven day embryonated

eggs for which three eggs were used for each dilution. An "x" indicates one day of survival. Eggs living to the twelfth day were discarded.

FO	xxxxxxx	FO	xx
FO	xxx	FO	xx
FO	x	FO	xx
FO	x		
FA	xxxxxxxxxxx	FA	xxxxxxxxxxx
FA	xxxxxxxx	FA	xxxxxxxxxxx
FA	xxxxxxxx	FA	xxxxxxxxx
FA	xxxxxxx		
FB	xxxxx		
FB	x		
FB	x		
FB	x		
FC	xxxxxxx		
FC	xxx		
FC	xx		
FC	x		
FD	xxxxxxx		
FD	xxxx		
FD	xxxx		
FD	xx		
F	xx	F	xxxxxx
F	x	F	xxxxxx
F	x	F	xxxxxx
F	x		

the ethyl alcohol. While these results are not clearcut, they indicate some control of the virus of feline pneumonitis by ethyl vanillate.

The first rickettsia tested was that of Q-fever. .1 cc. of the suspended rickettsia was injected into each egg. Dilutions A, B, C, and D of ethyl vanillate were injected in a series of eggs, six being used for each dilution. .1 cc. of the ethyl vanillate solution was injected into each of the eggs. The results of these tests are shown in Chart 5. The results do not indicate that ethyl vanillate has any appreciable effect in controlling Q-fever.

CHART 5. Chart showing the effect of inoculations of .1 cc. of a suspension of the rickettsia of Q-fever, with six eggs for the control and for each of the dilutions of ethyl vanillate used. The dilutions of ethyl vanillate used were A, B, C and D. All of the eggs were dead by the eighth day. An "x" indicates one day's survival of the egg.

QA	xxxxxxxx	QD	xxxxxx
	xxxxxx		xxxxx
	xxxxx		xxxxx
	xxxxx		xxxxx
	xxx		xxxx
	xxx		xxx
QB	xxxxxxx	QControl	xxxxxx
	xxxxx		xxxxx
	xxxxx		xxxx
	xxxx		xxx
	xxxx		xxx
	xxx		xx
QC	xxxxxx		
	xxxxx		
	xxxx		
	xxx		
	xxx		
	xxx		

The second rickettsia tested was that of rickettsial pox. .1 cc. of the suspended rickettsia was injected into each egg. Dilutions A, B, C, and D of ethyl vanillate were infected into a series of eggs, six being used for each dilution. .1 cc. of the ethyl vanillate solution was injected into each of the eggs. The results of these tests are shown in Chart 6. The results do not indicate that ethyl vanillate has any appreciable effect in controlling rickettsial pox.

CHART 6. Chart showing the effect of inoculations of .1 cc. of a suspension of the rickettsia or rickettsial pox into six eggs for the control and into six for each of the dilutions of ethyl vanillate used. The dilutions of ethyl vanillate used were A, B, C, and D. All of the eggs were dead by the eighth day. An "x" indicates one day survival of the egg.

RA	xxxxxxxx	RD	xxxxxx
	xxxx		xxxxxx
	xxx		xxxxxx
	xxx		xxxxx
	xxx		xxxx
	xxx		xxx
RB	xxxxxx	RControl	xxxxxx
	xxxx		xxxxx
	xxxx		xxxxx
	xxxx		xxxx
	xx		xxx
	xx		xx
RC	xxxxxx		
	xxxxx		
	xxx		
	xxx		
	xxx		
	xx		

From the results of this preliminary type of screen testing of embryonated eggs for the effectiveness of ethyl vanillate in the control of viruses and rickettsias, it would seem that .005 and .0005 g. of ethyl vanillate per egg have an inhibitory effect upon the growth of most of the viruses tested, but that these concentrations do not effect the growth of the rickettsias.

Ethyl Vanillate* Studies on Embryonated Eggs. III.

Robert W. Edwards and Fred A. Barkley

(*Hektoen Institute for Medical Research, Cook County Hospital, Chicago, Ill.*)

A series of four species of bacteria was used in a series of inoculations of ethyl vanillate in embryonated eggs to obtain an indication of its activity against them. The four were inoculated at about 10^7 bacteria per egg, using turbidity of the suspension for standardization. The dilutions of ethyl vanillate used were:

"A"	.05	g. of ethyl vanillate per cc. of 50	% ethyl alcohol
"B"	.005	g. "	"	"	"	" 15	%	"	"
"C"	.0005	g. "	"	"	"	" 1.5%		"	"
"D"	.00005	g. "	"	"	"	" .15%		"	"

The first bacterium tested was *Shigella Sonnei*. Six seven day embryonated eggs were inoculated with each dilution and six controls were run. The results of the inoculations are shown in Chart 1. It will be seen that the ethyl vanillate showed no particular effect on the killing time of *Shigella Sonnei* in embryonated eggs.

Chart 1. Showing the effects of inoculation of seven day embryonated eggs with 10^7 organisms of *Shigella Sonnei* with a series of dilutions of ethyl vanillate. Eggs living the thirteenth day were discarded. An "x" indicates one day of survival by an embryonated egg.

A	xxxxxxxxxxxx	D	xxxxxxxxxxxx
	xxxxxxxxxxxx		xxxxxx
	xxxxxxx		xxxxxx
	xxx		xxxxx
	xxx		xx
	xxx		xx
B	xxxxxxxx	Control	xxxxxxxxxxxx
	xxxxxx		xxxxxxxxxxxx
	xx		xxxxxxxx
	xx		xxxxxx
	xx		xx
	x		x
C	xxxxxxxxxx		
	xxxxxxxx		
	xxxxxx		
	xx		
	xx		
	xx		

The second bacterium tested was *Salmonella typhosa*. Six seven day embryonated eggs were inoculated with each dilution and six controls were run. The results of these are shown in Chart 2. It would seem

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that ethyl vanillate does have a slight effect in controlling this bacterial infection when inoculated into eggs.

CHART 2. Chart showing the effects of inoculation of seven day embryonated eggs with 10^7 organisms of *Salmonella typhosa* with a series of dilutions of ethyl vanillate. An "x" indicates one day of survival by an embryonated egg.

A	xxxxxxxxxxx	D	xxx
	xxxxxxxx		xx
	xxxxxx		xx
	xxx		xx
	xx		x
	x		x
B	xxxxx	Control	xxx
	xx		xx
	xx		x
	xx		x
	xx		x
C	xxx		
	xxx		
	xxx		
	xx		
	xx		
	x		

The third bacterium tested was *Salmonella typhimurium*. Six seven day embryonated eggs were used for controls and six were inoculated with each dilution. Chart 3 shows the results of the inoculations. It will be seen that ethyl vanillate does seem to have some effect in its higher concentration on the control of the bacterial infection of the egg.

CHART 3. This shows the effects of inoculation of seven day embryonated eggs with 10^7 organisms of *Salmonella typhimurium* with a series of dilutions of ethyl vanillate. Eggs living the thirteenth day were discarded. An "x" indicates one day of survival by an embryonated egg.

A	xxxxxxxxxxxx	D	xxxx
	xxxxxxxxxxxx		xxx
	xxxxxx		xx
	x		x
	x		x
	x		x
B	xxxx	Control	xxxx
	xxx		xx
	xx		xx
	x		x
	x		x
	x		x
C	xxx		
	xx		
	x		
	x		
	x		
	x		

The fourth bacterium tested was *Shigella paradysenteriae*. Six seven day embryonated eggs were inoculated with each dilution and six controls were run. The results of these inoculations may be seen in Chart 4. It would seem that ethyl vanillate in its higher concentrations does exert a slight control on the bacterial infection of this disease in embryonated eggs.

CHART 4. Chart showing the effects of inoculation of seven day embryonated eggs with 10^7 organisms of *Shigella* (Flexner II) with a series of solutions of ethyl vanillate. An "x" indicates one day of survival by an embryonated egg.

A	xxxxxxxxxxxx	D	xx
	xxxxxxx		xx
	xxxxxx		xx
	xx		xx
	xx		xx
	xx		x
B	xxxx	Control	xxxxxx
	xxx		xx
	xx		xx
	x		xx
	x		xx
	x		x
C	xxxxx		
	xxxx		
	xx		
	xx		
	xx		
	x		

In an attempt to see if there was any effect of acid-fast organisms .1 cc. of 1/200 mg. wt. per cc. of the *Mycobacterium* of tuberculosis (strain H37RV) was injected into seven day embryonated eggs. .5 cc. of a solution containing .1 g. ethyl vanillate per cc. of 20 percent ethyl alcohol (Solution "O"), .5 cc. of a solution containing ol. g. ethyl vanillate per cc. of 10 percent ethyl alcohol (Solution "A"), and a control without ethyl vanillate was used. Acid fast organisms were not obtained from smears made from the eggs when they died. The results are graphically shown in Chart 5. Since acid fast organisms were not

CHART 5. Table showing the results of the inoculation of eggs with suspensions of the tuberculosis (strain H37RV) bacillus into seven day embryonated eggs with two dilutions of ethyl vanillate. An "x" indicates one day of survival of the embryonated egg.

O	xxxxxxxxxx	A	xx	Control	xxxxxxx
	xxxxxxx		x		xxxxxx

obtained from the dead eggs, one cannot draw any conclusions from the results. In a further attempt to find if ethyl vanillate has any effect on acid fast organisms, three eggs were inoculated with .1 cc of a suspension of culture of acid-fast organisms. At the end of five days all three eggs were alive. On the sixth day one of the three eggs was injected with .1 cc. of a solution containing 1 gram of ethyl vanillate per cc. of peanut oil. The two not injected with peanut oil died after four days and smears made from them showed acid fast organisms, while the one injected with the ethyl vanillate in peanut oil survived nine days and acid fast organisms were not found in smears made from it.

From the small series of tests made with bacteria and ethyl vanillate in embryonated eggs, it would appear that in some cases there is a sufficient inhibitory effect on the bacteria by the higher concentrations of the ethyl vanillate to indicate that further studies should be made both with other bacteria in embryonated eggs and with experimental mammals.

Ethyl Vanillate* Studies on Embryonated Eggs. IV.

ROBERT W. EDWARDS AND FRED A. BARKLEY

(Hektoen Institute for Medical Research, Cook County Hospital, Chicago, Ill.)

In an attempt to find the activity of ethyl vanillate on fungal activity in embryonated eggs a series of inoculations was made using various dilutions of ethyl vanillate and various fungi. The dilutions of ethyl vanillate used were

"O"	.5	g.	of	ethyl	vanillate	per	cc.	of 95 %	ethyl	alcohol
"A"	.05	g.	"	"	"	"	"	50 %	"	"
"B"	.005	g.	"	"	"	"	"	15 %	"	"
"D"	.00005	g.	"	"	"	"	"	.15%	"	"

The first fungus used was *Aspergillus* sp. .1 cc. of each dilution of ethyl vanillate and .1 cc. of a suspension of the spores was used to inoculate each egg. The results of the inoculations are shown in Table 1.

TABLE 1. Chart showing (on the left) the effects of inoculating eggs with a suspension of *Aspergillus* spores and various dilutions of ethyl vanillate, (in the center) the effects of inoculating eggs with a suspension of *Aspergillus* spores and various dilutions of ethyl vanillate, and (to the right) the effects of inoculating eggs with a dilute suspension of *Aspergillus* spores (10^{-6} concentration of that used in the other two tests) and a dilution of ethyl vanillate in peanut oil (see text).

A	x	O	xx	EV	xxxxx
	x		xx		xxxxx
	x		xx		xxx
B	xx	A	xxx		xxx
	xx		xx		xxx
	xx		xx		xxx
D	xx			Control	xxx
	xx				xxx
	x				xxx
Control	x	Control	xx		xxx
	x		xx		
	x		xx		

Another inoculation was made using .1 cc. of a suspension of the spores, .1 cc. of the dilutions "O" and "A" and .3 cc. of sterile water. The results of these are shown in Table 1. Another inoculation was made using .2 cc. of a suspension of spores in each egg, with four controls run and six eggs receiving .1 cc. of 2. g. ethyl vanillate per cc. of peanut oil. The results of this inoculation are shown in Table 1.

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*Supplied by The Institute of Paper Chemistry, Appleton, Wis. Supported by a grant from the Dr. Leonard H. and Louis Weissman Medical Research Foundation.

The second fungus used was *Geotrichum* sp. .1 cc. of each dilution of ethyl vanillate and .1 cc of a twenty-four hour Sabouraud's acid broth (pH 4) culture were inoculated into seven day embryonated eggs. The results of the inoculations are shown in Table 2

TABLE 2. Chart showing the effects of inoculating eggs with a culture of *Geotrichum* sp. and a series of dilutions of ethyl vanillate in seven day embryonated eggs. Each "x" indicates one day of survival of the embryo. Eggs living to the seventh day were discarded.

O	xxxx	Control	xxxxxxx
	xxx		xxxxxx
	x		xxxx
A	xxxxxxx		
	xxxxxxx		
	xxxxxx		

The third fungus tested was *Trichoderma* sp. .1 cc. of two dilutions of ethyl vanillate and .1 cc. of a twenty-four hour Sabouraud's acid broth (pH 4) culture were inoculated into seven day embryonated eggs. The results of the inoculations are shown in Table 3.

TABLE 3. Chart showing the effects of inoculating seven day embryonated eggs with a culture of *Trichoderma* sp. and two dilutions of ethyl vanillate. Each "x" indicates one day of survival of the embryo. Eggs living to the seventh day were discarded.

O	xxxxxxx	A	xxxxxx	Control	xxxxxxx
	xxxxxxx		xxxxxx		xxxxxxx
	xxxxxx		xxxxx		xxxxx

The fourth fungus tested was *Trichophyton* sp. .1 cc. of two dilutions of ethyl vanillate and .1 cc. of a twenty-four hour Sabouraud's acid broth (pH 4) culture were inoculated into seven day embryonated eggs. The results of the inoculations are shown in Table 4.

TABLE 4. Chart showing the effects of inoculating with a culture of *Trichophyton* sp. and a series of dilutions of ethyl vanillate using seven day old embryonated eggs. Each "x" indicates one day of survival of the embryo. Eggs living to the eighth day were discarded.

O	xxxxx	A	xxxxxxx	Control	xxxxxxxx
	xx		xxxxxxx		xxxxxxx
	x		xxxxxxx		xxxxxx

The fifth fungus tested was *Candida tropicalis*. .1 cc of three dilutions of ethyl vanillate and .1 cc. of a twenty-four hour Sabouraud's acid broth (pH 4) culture were inoculated into seven day embryonated eggs. This was repeated using "O" and "A" dilutions of ethyl vanillate. Another inoculation was made using .2 cc. of a 10^{-6} dilution acid broth culture for each egg, with four controls and six eggs receiving .1 cc. of a .2g ethyl vanillate per cc. of peanut oil. The results of these inoculations are shown in Table 5.

TABLE 5. Chart showing (on the left) the effects of inoculating eggs with an acid broth culture of *Candida tropicalis* and various dilutions of ethyl vanillate (in the center), the effects of inoculating eggs with an acid broth culture of the

same species with two dilutions of ethyl vanillate and (to the right) the effects of inoculating eggs with a 10^{-6} dilution of acid broth culture and a dilution of ethyl vanillate in peanut oil (see text). (All four of the controls of the 10^{-6} dilutions of acid broth culture eggs when stabbed on cornmeal agar were positive for Monilia while only three of those treated with ethyl vanillate were positive.)

A	x	O	xx	EV	xxxxxxxxx
	x		x		xxxxxxxx
	x		x		xxxx
B	xxx	A	xx		xxxx
	xx		xx		xxx
	x		x		xxx
D	xxxxxxxxx				xx
	xx			Control	xxxxxxxxx
	xx				xxxxxxxxx
Control	xxxxxxxxx	Control	xxxxx		xxx
	xxx		xx		xxx
	x		x		

The sixth fungus tested was *Candida albicans.* This was tested parallel to the tests of *Candida tropicalis.* The results of these inoculations are shown in Table 6.

TABLE 6. Chart showing (on the left) the effects of inoculating eggs with an acid broth culture of *Candida albicans* and three dilutions of ethyl vanillate, (in the center) the effects of inoculating eggs with an acid broth culture of the same species with two dilutions of ethyl vanillate and (to the right) the effects of inoculating eggs with a 10^{-6} dilution of acid broth culture and a dilution of ethyl vanillate in peanut oil (see text). (None of the controls produced mycelia upon being stabbed into cornmeal agar and only one of the ethyl vanillate series.) Eggs living to the twelfth day were discarded.

A	x	O	xxxxxxx	EV	xxxxx
	x		xxxxx		xxxxx
	x		xxx		xx
B	xxxxxxxxxxx	A	xxxxxx		xx
	xxxxxxxxxxx		xxxxxx		x
	x		xxxx		x
D	xxxxxxxxxxx			Control	xxxxxxxxxxx
	xxxxxxxxxx				xxxxxxxxxxx
	xxxxxxxxxx				xxxxxxxxxxx
Control	xxxxxxxxxxx	Control	xxx		xxxxxxxxxxx
	xxxxxxxxxx		xxx		
	xxxxxxxxxx		xx		

In the series where the three dilutions of ethyl vanillate were inoculated the lethal effect was due to the action of the ethyl alcohol apparently, for the series where only two dilutions were used, these were mixed with three parts of water to one of the ethyl vanillate and .4 cc. was injected (giving thereby the same quantity of ethyl vanillate in more dilute alcohol with much less toxic effects). The peanut oil solutions of ethyl vanillate seemed to increase the toxicity of the fungus, although when injected without the fungi it is almost without toxicity.

The ethyl vanillate studies in relation to controlling fungi were quite disappointing, indicating only slight control of their growth in eggs. It is possible that solvents other than those used would give more promising results.

Ethyl Vanillate* Studies on Embryonated Eggs. V.

Fred A. Barkley and Robert W. Edwards
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In an attempt to find the activity of ethyl vanillate on the growth of Toxoplasma in embryonated eggs a series of inoculations was made using various dilutions of ethyl vanillate and the abdominal fluid from infected mice.

The dilutions of ethyl vanillate used were:

"O"	.5	g.	of	ethyl	vanillate	per	cc.	of	95 %	ethyl	alcohol
"A"	.05	g.	"	"	"	"	"		50 %	"	"
"B"	.005	g.	"	"	"	"	"		15 %	"	"
"D"	.0005	g.	"	"	"	"	"		.15%	"	"
"E"	.2	g.	"	"	"	"	"		peanut oil.		

.1 cc. each of two solutions of ethyl vanillate was used in a series of eggs which, along with the controls, were inoculated with .1 cc. of 15% fluid from the abdomen of infected mice in saline penicillin solution. The results are shown in Table 1.

Table 1. Showing the effect of inoculating a series of seven day embryonated eggs with .1 cc. of a fifteen percent fluid suspension from the abdomen of infected mice in saline penicillin solution with two dilutions of ethyl vanillate. Each "x" indicates one day of survival of the embryo.

B	D	Control
xxxxxx	xx	xx
xx	x	x
xx	x	x

.1 cc. each of two solutions of ethyl vanillate was used in a series of eggs which, along with the controls, were inoculated with .1 cc. of 15% fluid from the abdomen of infected mice suspended in saline penicillin solution. The survival time for these eggs is shown in Table 2. Since it is difficult to read the smears of material taken from eggs, .1 cc. of material from each egg as it died (or was discarded on the twelfth day) from the two surviving the longest of each set was injected into a mouse. The mice inoculated from the control eggs died in four days and their abdominal fluid showed an abundance of Toxoplasma, while the mice inoculated from the ethyl vanillate treated eggs did not die for two weeks, at which time they were discarded.

Table 2. Showing the effect of inoculating a series of seven day embryonated eggs with .1 cc. of a fifteen percent fluid suspension from the abdomen of infected mice in saline penicillin solution with two dilutions of ethyl vanillate. Each "x" indicates one day of survival of the embryo.

O	A	Control
xxxxxxxxxxxx	xxxxxxxxxxxx	xxxxxx
xxxxxxxxxxxx	xxxxxxxxxxxx	xxxxxx
xxxxx	x	x

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A 10^{-6} dilution of the abdominal fluid of mice infected with Toxoplasma was inoculated into four eggs in the amount of .2 cc. per egg. The same amount of abdominal fluid of the same dilution was placed in six other eggs and .1 cc. of ethyl vanillate in peanut oil was injected. The results are shown in Table 3.

TABLE 3. Chart showing the effect of inoculating a series of eggs with 10^{-6} dilution of abdominal fluid of mice infected with Toxoplasma and ethyl vanillate in peanut oil. Each "x" indicates one day of survival of the egg. Eggs living ten days were discarded.

EV	xxxxxxxxxx	Control	xxx
EV	xxxxxxxxx		xxx
	xxxxxxxxx		xx
	xxx		x
	xxx		
	xxx		

From the results of these inoculations it would seem that ethyl vanillate has a decided effect in controlling Toxoplasma infection in embryonated eggs. Limited studies by the authors, using ethyl vanillate on mice infected with Toxoplasma, have not been particularly promising.

Ethyl Vanillate* Studies on Embryonated Eggs. VI.

Ernest Hartman and Fred A. Barkley

(*Stritch School of Medicine, Loyola University, and Hektoen Institute for Medical Research, Cook County Hospital, Chicago, Ill.*)

In an attempt to find if ethyl vanillate has a controlling effect on *Trichomonas foetus*, .1 cc. of a suspension was injected into the amniotic cavity of a series of five eggs, into two of which .1 cc. of a peanut oil solution of ethyl vanillate (1 gram of ethyl vanillate in 5 cc. of peanut oil) was injected into the allantoic cavity. This was repeated with the suspension of *Trichomonas* diluted 10^{-1}. The results of the test in terms of survival of the embryos are indicated in Table 1. Living and active *Trichomonas* were found in the amniotic fluid of the control eggs as they died while none were found in those treated with ethyl vanillate in peanut oil.

Table 1. The survival results of inoculating *Trichonomas* cultures (to the left) and of cultures diluted 10^{-1} (to the right) into the amniotic cavities of embryonated eggs and of inoculating dilute ethyl vanillate in peanut oil into the allantoic cavity. Each "x" indicates one day of survival of the embryo. Eggs living to the tenth day were sacrificed for examination for the presence of *Trichomonas*.

EV plus Trichomonas 10^{-0}	xxxxxxxxxx	EV plus Trichomonas 10^{-1}	xxxxxxxxxx
	xxxx		xxxxxxxxxx
Control	xxxxx	Control	x
	x		x
	x		x

From the present test it would appear that ethyl vanillate exerts some control over the persistence of *Trichomonas* in embryonated eggs.

*Supplied by The Institute of Paper Chemistry, Appleton, Wis. Supported by a grant from the Dr. Leonard H. and Louis Weissman Medical Research Foundation.

The Vertical Distribution of Certain Entomostracans in Sodon Lake

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INTRODUCTION

The study reported upon here represents one phase of a seasonal limnological investigation of Sodon Lake. This lake, located in Oakland County, Bloomfield Hills, Michigan, is small (approximately 5.9 acres in surface area), of glacial origin and has a maximum depth of about 57 feet. The two main characteristics of the lake are: an anaerobic condition throughout the year below the 25 foot level, and a rather low concentration of hydrogen sulfide emanating from the bottom deposits. A detailed description of the morphometric conditions of Sodon Lake is given by Newcombe and Slater (1949).

It is the purpose of this paper to present quantitative data showing the seasonal, vertical distribution of certain members of the zooplankton and to discuss the possible relationship of selected chemical, physical, and biological factors to this plankton.

METHODS

Observations were made during the interval February 19, 1949 to November 15, 1949; fortnightly zooplankton samples[2] were taken from a permanent station, located approximately in the center of the lake and at its greatest depth; it is logical to assume that, considering the morphometric conditions of Sodon Lake, the samples secured were representative of the euplankton.

Samples were taken at 1, 3 and 5 foot depths; thereafter the samples were obtained at 5 foot intervals up to and including 40 feet. There were no indications of animal life existing beyond the 40 foot zone. One liter samples were obtained, using a Kemmerer water bottle; the samples were then strained through a number 25 silk plankton net and transferred to small vials. In the laboratory all organisms were checked for viability; all viable organisms were preserved in 70% alcohol for enumeration and expressed in numbers per liter.

Phytoplankton samples were secured at the same times and under identical conditions except that a Forest Centrifuge was used to obtain the concentrate; 3 liter phytoplankton samples were usually secured. Water samples for chemical and bacteriological analyses were obtained simultaneously with the plankton samples.

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A comment is indicated here concerning the supposed difficulties inherent in the obtaining of a water sample from a specific zone and, at the same time, insure that the sample is representative of that particular zone and not contaminated by extraneous matter. In preliminary work concerned with a bacteriological investigation of Sodon Lake, samples were obtained in sterile glass bottles fitted with inlet tubes which were broken at the desired depth by sending down a messenger. However, neither qualitatively nor quantitatively did the populations differ from those obtained by using the Kemmerer sampler. For the reason expressed, it would appear that there is justification for using the metal sampling bottle and that the populations to be noted are representative of the indicated depths.

The chemical data to be presented are based upon spectrographic analyses of 3 liter samples; these were secured under the same conditions as the zooplankton samples. The rapid Winkler method of oxygen determination was used throughout this investigation. The procedure up to and including acidification was carried out in the field and titrations were effected immediately upon return to the laboratory. Concentrations of solutions were such that the final titration in c.c. was equal to parts per million of dissolved oxygen, Theriault (1925). Hydrogen sulfide was determined by the method of Theroux, Eldridge and Mallmann (1936).

Temperatures were taken with a Foxboro Thermophone. Light penetration through the water and ice was measured with a specially designed submersible photometer; essentially the apparatus is the same as used by Greenbank (1945) in his limnological investigations of ice-cover lakes as related to winter-kill of fish. All pH values were measured electrometrically, using a Leeds and Northup pH meter.

OBSERVATIONS

In general it is true that the zooplankton of Sodon Lake is dominated by three entomostracans, *Daphnia pulex*, *Diaptomus oregonensis* and *Cyclops prasinus;* other plankters, *Daphnia longispina* and *Bosmina coregoni* have appeared but in such extremely small numbers that they may be considered as transient members of the plankton community. Whenever the transient forms have been noted in the plankton, their presence has been confined to 15 ± 5 feet. Members of the zooplankton, other than those indicated above and nauplii of the copepods have been virtually non-existant. The three dominant members of the zooplankton indicated above have been arranged according to depth and season in Tables 1, 2, and 3.

In tracing the seasonal periodicity of the individual zooplankters under the conditions of this experiment, we are actually tracing the seasonal history of the total net plankton as the net plankton is limited to the entomostracans listed above.

Under the conditions reported upon here, the zooplankton shows pronounced increases in numbers during March, May, June and September; several minima can also be observed (Fig. 1). Pennak (1946), in discussing the dynamics of production in aquatic populations, draws attention to the fact that, while the total Entomostraca curve is generally thought to follow the typical bimodal annual curve, the

TABLE 1.—*Numbers per liter of Daphnia pulex, by season and depth, Sodon Lake, 1949.*
Samples taken about 11:00 A. M.

Depth per ft.	DATE																	Totals
	2/19	3/5	3/19	4/2	4/16	4/30	5/14	5/28	6/11	6/25	7/9	7/23	8/4*	8/20	9/3	9/17	10/1	10/15	10/29	
1	n.s.	n.s.	n.s.	4	21	3	n.s.	25	....	....	3	n.s.	4	12	n.s.	6	1	5	1	89
3	37	21	14	10	1	37	21	16	1	3	3	1	14	1	4	12	11	12	1	220
5	37	13	53	9	51	35	16	24	11	....	3	3	14	1	9	24	13	10	2	328
10	8	22	59	27	64	48	3	19	12	5	19	26	11	1	6	56	27	26	4	392
15	12	6	18	3	22	54	20	9	13	31	22	18	19	n.s.	8	68	28	44	36	431
20	15	4	19	....	5	5	7	3	33	....	13	27	12	n.s.	10	19	22	13	24	245
25	....	1	1	....	6	37	....	2	....	5	....	9	5	n.s.	7	5	8	3	3	82
30	....	2	1	....	2	....	2	5	2	1	2	2	1	n.s.	8	2	4	3	4	41
35	....	....	....	2	38	3	1	....	....	1	3	2	....	n.s.	1	....	1	4	1	57
40	....	....	....	2	5	1	....	....	1	3	....	....	2	n.s.	1	1	....	2	....	19
	109	69	166	57	215	223	70	103	73	49	68	88	82		54	193	115	122	76	1,899

* Samples taken at 2:00 P. M.

TABLE 2.—*Numbers per liter of Cyclops prasinus, by season and depth, Sodon Lake, 1949.*
Samples taken about 11:00 A. M.

Depth in Ft.	DATE																		Totals
	2/19	3/5	3/19	4/2	4/16	4/30	5/14	5/28	6/11	6/25	7/9	7/23	8/4*	8/20	9/3	9/17	10/1	10/15	10/29	
1	n.s.	n.s.	n.s.	.....	.....	2	n.s.	7	1	.....	1	n.s.	1	1	n.s.	9	14	5	4	45
3	.....	.....	1	.....	.....	7	10	12	4	1	.....	.....	2	1	5	11	14	8	16	92
55	.....	.....	2	.....	2	9	13	16	8	3	1	.....	1	.....	5	18	4	16	12	110
10	.....	1	10	4	2	4	10	15	20	5	4	2	4	.....	1	12	8	5	14	121
15	.....	1	28	2	3	11	15	15	19	11	6	.....	10	n.s.	2	11	6	14	9	163
20	.....	11	57	7	2	2	8	4	49	21	9	19	7	n.s.	.....	2	5	10	6	219
25	.....	14	11	2	.....	12	1	2	3	2	3	9	1	n.s.	2	.....	.....	1	1	64
30	.....	1	1	2	1	1	.....	4	3	1	3	.....	1	n.s.	.....	.....	3	.....	2	23
35	.....	.....	1	2	.....	.....	2	1	2	2	1	.....	1	n.s.	1	.....	.....	.....	.....	13
40	.....	.....	.....	.....	.....	.....	.....	2	1	1	.....	1	.....	n.s.	.....	.....	.....	.....	1	6
	0	28	111	19	10	48	59	78	110	47	28	31	28		16	63	54	59	65	856

* Samples taken at 2:00 P. M.

TABLE 3.—*Numbers per liter of Diaptomus oregonensis, by season and depth, Sodon Lake, 1949.*
Samples taken about 11:00 A. M.

Depth in Ft.	DATE																		Totals
	2/19	3/5	3/19	4/2	4/16	4/30	5/14	5/28	6/11	6/25	7/9	7/23	8/4*	8/20	9/3	9/17	10/1	10/15	10/29	
1	n.s.	n.s.	n.s.	1	3	3	n.s.	11	1	....	5	n.s.	11	1	n.s.	13	2	8	....	59
3	2	3	1	3	1	4	2	16	....	2	3	1	12	....	2	18	7	12	5	94
5	2	1	2	2	3	8	2	13	2	6	4	....	9	3	7	15	4	17	13	113
10	3	15	18	4	4	11	3	26	....	5	8	8	2	6	3	5	6	8	8	143
15	3	8	2	1	1	16	18	14	12	13	12	7	2	n.s.	2	4	2	7	2	126
20	2	4	....	1	....	3	9	3	43	15	9	6	2	n.s.	1	4	1	1	1	105
25	....	4	1	1	2	8	1	2	....	1	2	....	....	n.s.	....	1	....	1	....	24
30	1	....	....	....	....	....	....	2	2	....	....	1	1	n.s.	11	....	....	2	....	10
35	....	....	....	....	....	....	1	1	3	....	1	....	1	n.s.	....	2	....	1	....	14
40	....	....	....	1	3	....	1	1	2	....	....	1	....	n.s.	....	1	....	....	....	5
	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
	13	35	24	14	17	53	36	89	65	42	44	24	40		16	63	22	57	29	693

* Samples taken at 2:00 P. M.

classical curve is based upon data from comparatively few deep lakes and rivers. Pennak, loc. cit., also states that, for the Entomostraca population of the 7 Colorado lakes which he investigated, the pulses may occur at "almost any time of the year." Our observations on Sodon Lake over a twelve month period seem to support Pennak's contention. In reference to the phytoplankton it is again usually stated that the annual curve either approximates that of the zooplankton or coincides with it. Our data on the phytoplankton of Sodon Lake indicate that the phytoplankton, exclusive of bacteria, was almost non-existant until late March. At this time we were able to demonstrate a phytoplankton count of approximately 9,000 per liter for the following organisms: *Schroederia setigera*, *Oscillatoria tenuis*, *Eudorina sp.*, and *Staurastrum sp.* This fact is of particular interest when an explanation of the nutrition of the entomostracans is sought.

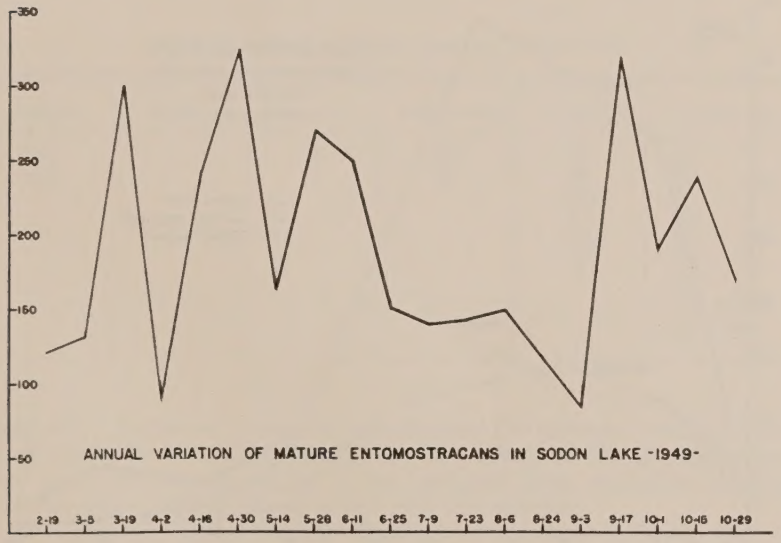

FIG. 1. Annual variation of mature entomostracans in Sodon Lake in 1949.

It is usually stated or at least implied that the various chemical and physical factors operating on a body of water control the quality and quantity of the plankton; among others, the factors of hydrogen-ion concentration, dissolved oxygen, temperature and inorganic nutrients are included. However, Hutchinson (1944) states: "Clear cut correlations between chemical conditions and the qualitative composition of the phytoplankton (and zooplankton) are not to be expected." This concept may well be extended, in the present investigation, to include the quantitative composition of the plankton as well. We have previously indicated that the transient members of the zooplankton appear at 15 ± 5 feet. The maximum numbers of the resident population also appear at this depth, Fig. 2.

The pH at any particular level of Sodon Lake tends to change but little throughout the year; also the values indicate but slight change in vertical distribution. The hydrogen-ion concentration is always in the alkaline range (pH 7.35–7.0) in the epilimnion and thermocline regions. The maximum vertical range is approximately 1.1 pH units. It seems probable that pH, per se, can be of little significance in regulating the distribution under consideration.

We attach slight, if any, importance to temperature as a governing factor for the distributions under investigation. Let us consider again the fact that the majority of the entomostracans are found at 15 ± 5 feet. At these depths, we have, to cite just two examples, a range of 10.1° in May and a range of 27.5° in August. Temperature obviously can not, in itself, be an important controlling factor for the distributions under consideration.

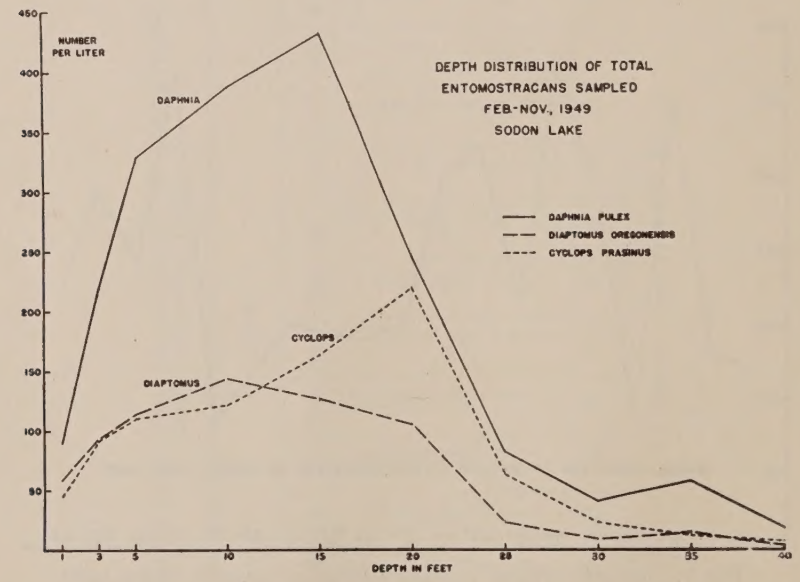

FIG. 2. Depth distribution of total entomostracans sampled Feb.–Nov., 1949, in Sodon Lake.

While the dissolved oxygen content is never considered to be a limiting factor in an unpolluted body of water, Sodon Lake does exhibit some interesting oxygen relationships. Newcombe (1950) has reported that in detail on these phenomena. He concluded that, under summer conditions, approximately 35% of the total volume of Sodon Lake was anaerobic and that at 16 feet there was always less than 1 p.p.m. of dissolved oxygen. He further stated, loc. cit., that, during the winter, no dissolved oxygen existed below 24 feet. We have been unable, throughout the present investigation, to obtain a positive dissolved oxygen test below 25 feet. However, it will be recalled that the bulk of the plankton population is found at about 20 feet where the oxygen values seem to be adequate for the plankton entomostraca. The

annual range of dissolved oxygen at 20 feet has been from 5 p.p.m. to 0.46 p.p.m. These oxygen values are noticeably less than 0.2 cc. per liter which according to Pennak (1946) is the critical point for most plankton entomostracans. At any rate, it must be concluded that the entomostracans under investigation show an extremely high tolerance to marginal amounts of oxygen and that the figure cited by Pennak must be modified, at least, for Sodon Lake. In this connection, it should be emphasized that the entomostracans also show considerable tolerance to hydrogen sulfide as measurable amounts of this gas are found just below 25 feet, Bicknell (1949), Newcombe (1950).

Almost nothing is known regarding the light requirements of the plankton of inland lakes. It is entirely possible that each individual species has its own toleration point. A comment is included here only to emphasize the point that below 25 feet 0.0% penetration was always

TABLE 4.—*Spectrographic Analysis, Sodon Lake.*

Major	Minor	Low	Very Low	0.01%	0.001%
Mg		B	FE	P	Ni
Ca			Si	Al	Bi
				Cu	Cr
				Ti	Mo
				Mn	Co
				V	Pb
					Sn
					Na trace
					Ag trace

obtained. Inasmuch as but few members of the plankton were obtained much below 25 feet, this figure limits the extent of the photosynthetic zone and, with the exception of the bacterial flora, probably coincides with the lower limit of the zone of biological activity.

In Table 4 is presented the chemical composition of Sodon Lake as determined by the spectrochemical method. The concentrations reported do not differ for any time of the year or for any depth. All computed amounts may be included in 6 categories indicated as follows: major components (over 10%), minor (1–10%), low (0.1–1.0%), very low (0.1%), below 0.01% and below 0.001%.

It is to the biological factors that we turn for a possible explanation of the distribution of the entomostracans. It has been emphasized that the zooplankton approached a numerical peak before the phytoplankton appeared in measurable numbers. It would then appear that the entomostracans were independent of the algal flora for food and that they must turn either to colloidal material present in the water or to the bacteria. This idea is not new and has previously been advanced by Huff (1923). From our bacteriological studies Bicknell (1949), we have considerable evidence that the greatest concentration of bacteria exists just below 20 feet and that their numbers diminish as the vertical

distance becomes less. It would then appear that there is circumstantial evidence, at least, that there is a definite nutritional response by the copepods to the bacterial flora in their immediate environment.

SUMMARY

The qualitative and quantitative composition of the zooplankton of Sodon Lake from February 15, 1949 to November 15, 1949 is presented. The factors of hydrogen-ion concentration, temperature, inorganic nutrients and dissolved oxygen are briefly discussed as related to the distribution of the zooplankton. Some evidence is presented to show that the plankton Entomostraca are probably nutritionally independent of the algal flora.

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